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(54) Title: IDENTIFICATION AND CLONING OF THE RECEPTOR GENE FOR SYMBIOTIC NITROGEN FIXATION

(57) Abstract: The invention relates to the <I>Medicago sativa</I> DNA sequence encoding the NORK polypeptide as well as to the protein determined by this DNA sequence. In another aspect, the invention relates to conserved DNA regions, primers, DNA probes, polypeptides and corresponding antibodies characteristic to the NORK gene family, as well as to methods and their utilization to isolate additional NORK genes and proteins from different legumes or other plants, and their use for producing transgenic plants. In another aspect, the invention relates to a method of cloning a DNA sequence encoding a function involved in one of the steps of symbiotic nitrogen fixation, as well as to methods of producing transgenic plant cells and cloned entire plants wherein the biosynthesis of the NORK polypeptide takes place. The invention also relates to the identification of a gene present in leguminous plants, encoding a function indispensable for symbiotic nitrogen fixation, to the sequencing of the gene, confirmation of the biological function of the gene, and the transformation of the gene into plants unable for symbiotic nitrogen fixation.

Identification and cloning of the receptor gene for symbiotic nitrogen fixation

TECHNICAL FIELD

The invention relates to the *Medicago sativa* DNA sequence encoding the NORK polypeptide as well as to the protein determined by this DNA sequence. In another aspect, the invention relates to conserved DNA regions, primers, DNA probes, polypeptides and corresponding antibodies characteristic to the NORK gene family, as well as to methods and their utilization to isolate additional NORK genes and proteins from different legumes or other plants, and their use for producing transgenic plants.

In another aspect, the invention relates to a method of cloning a DNA sequence encoding a function involved in one of the steps of symbiotic nitrogen fixation, as well as to methods of producing transgenic plant cells and cloned entire plants wherein the biosynthesis of the NORK polypeptide takes place.

The invention also relates to the identification of a gene present in leguminous plants, encoding a function indispensable for symbiotic nitrogen fixation, to the sequencing of the gene, confirmation of the biological function of the gene, and the transformation of the gene into plants unable for symbiotic nitrogen fixation.

BACKGROUND ART

It is well known that symbiotic nitrogen fixation covers the requirements of the nitrogen demands of a plant and ensures normal growth, development and seed production in soils with limited amount of fixed nitrogen without added artificial nitrogen fertilizers (1). Plants capable of symbiotic fixation differ from other plants in that they carry all of the genetic determinants in their genome which are needed for the biological process of symbiotic nitrogen fixation. For those experts

who are familiar with the recombinant DNA techniques it is obvious that these genes can be identified, isolated and transferred to other plants on such a way that they retain their function. In theory, all genes required for symbiotic nitrogen fixation can be transferred to recipient plants which eventually will be able to establish symbiotic nitrogen fixation, consequently, they can be grown on a more economical and environmental friendly manner.

The identification of genes involved in symbiotic nitrogen fixation are being studied very intensively worldwide, still the nucleotide and amino acid sequences which are presented in this patent application are completely novel, we had access only to randomly sequenced cDNA sequences.

The symbiotic nitrogen fixation is a biological process established between particular dicotyledonous plants and soil bacteria (2, 3). The symbiotic nitrogen fixation takes place mostly in the root system where root nodules develop, and are invaded by the bacteria. During the development of root nodules bacteria enter the plant cells and change to bacteroids, and in such form they fix nitrogen that is they convert the inert dinitrogen of the air into ammonia. This ammonia (which is a form of the so-called fixed nitrogen) can be utilized by the plants for their development. Since no artificial nitrogen fertilization is needed, symbiotic nitrogen fixation is an environmental friendly, cost effective and improve the quality of the soil, therefore, these plants are very suitable as green crops. Agricultural legumes, which are capable to fix the nitrogen from the air, are therefore very important part of the agriculture. Their seeds and green mass contribute to large extent to the human and animal amino acid and oil supply, therefore, legumes are extremely important in the worldwide food supply.

Some soil bacteria, like *Rhizobia* and *Actinomycetes*, are able to establish symbiotic nitrogen fixation relationship with given plants, like many legumes, *Trema*, *Casuarina*, *Alnus*, *Parasponia* (2, 3).

As a consequence of the evolution this symbiotic relationship is strictly species specific, a given group of bacteria establishes symbiosis only with a given group of plants (2, 3, 4, 5). Such symbiosis is established between *Sinorhizobium meliloti* and *Medicago*, *Melilotus*, *Trigonella* species (6), between *Rhizobium leguminosarum* bv. *viciae* and *Vicia* species, between *Rhizobium leguminosarum* bv. *trifolii* and given members of *Trifolium* species, between *Rhizobium etli* and *Phaseolus* species, between *Bradyrhizobium japonicum* and *Glycine max*, between *Azorhizobium caulinodans* and *Sesbania rostrata*, between *Mesorhizobium loti* and *Lotus japonicus* (7).

The biogenesis and development of the symbiotic nodule, as well as the invasion of the plants cells by the bacteria is a complex, multi-step process taking place through series of gene-interaction involving both bacterial and plant symbiotic genes. The formation of the nodule, which is inevitable for the symbiotic nitrogen fixation according to our present knowledge, starts to develop after a specific signal exchange between the microsymbiont (the bacteria) and the macrosymbiont (the plants). Through the root system, the macrosymbionts excrete specific flavonoid and other molecules into the environment, which are characteristic to and determined by the genetic material of the plant species (8, 9). As a response to these excreted molecules compatible bacteria approach by chemotaxis the plant root system (10) and start to produce a signal molecule, the so-called lipo-chitooligosaccharides abbreviated as Nod factors, which in turn are specific nodulation signal for the plant (11, 12, 13). During nodule formation compatible bacteria attach to the root-hair surface on a polar manner. In this joining special molecules like bacterial pili, plant lectins and other adhesive molecules are active components (14). Upon the action of the symbiotic specific Nod factors, characteristic plant responses occur like root-hair deformation, curling and branching (15). Inside the root tissue, the cortical cells start to divide. As a response of the specific Nod factor produced by the compatible bacteria a symbiotic nodule start to develop on the root or on the stem depending on the type of symbiosis. Some of the specific Nod factors are shown in Fig. 1. without the sake of completeness (5, 7). As a consequence of the root-hair curling

provoked by the specific Nod factor, the bacteria become surrounded and enter the so-called infection threads produced by the plant. The infection threads start to grow towards the core tissue of the plant, where they start to branch. Inside the infection thread, the bacteria divide and move along the infection threads. At the same time the cortical cells continue to divide and the nodule meristeme is formed. The branching infection threads enter the dividing cells and the bacteria are released inside the plants cells, where they continue to divide and in a short time they occupy most of the plant cytoplasm establishing the so-called infected cells. In the infected cells bacteria are surrounded by plant plasma membrane (peribacteroid membrane) in which the bacteria change to bacteroids, in which form they are capable to fix nitrogen (16, 17). The derepression of the nitrogenase enzyme complex takes place only in the bacteriods. The bacteroids, with the help of their nitrogenase enzyme complex, can reduce the inert dinitrogen into ammonia, the so-called combined nitrogen, which can be utilized by the macrosymbiont. Most of the ammonia formed during nitrogen fixation (that part which is not utilized by the bacteroids) is excreted by the bacteroids. The excreted ammonia is taken up by the nodule cells and transferred towards the vascular tissue by a proper conducting mechanism. The fully developed and active nodules are able to supply the plants with enough combined nitrogen which is needed for their normal growth and development.

Root-hair deformation and cortical cell division can be evoked by very low (10^{-8}-10^{-12} M) concentration of the compatible Nod factor even if the bacteria are not present, if the Nod factor is applied to the root. Following Nod factor treatment normal nodule structures are formed on the plant roots, in which no bacteria are present. From this phenomenon it is obvious, that the Nod factors are recognized by the root cells, which in turn start the specific signal transduction pathway leading to nodule formation. The recognition of the excreted Nod factor on the surface of the root cells is taking place by an unknown mechanism. Nod factor binding receptors were looked for by biochemical methods, but no such a supposed protein responsible for the binding and transmission of the Nod factor could have been identified so far (18). However, it is generally accepted, that the

bacterial Nod factor is recognized and bound by a specific receptor and through this mechanism the signal transduction cascade evokes nodule induction and development.

The steps leading to nodule development, the functions involved, and the genes coding for these functions in the plants are not known at present, however, one can understand that the presence of the microsymbiont for the formation of the nodule structure is not necessary. Some plants like alfalfa (*Medicago sativa* L.) is able to form spontaneous nodules. Under sterile conditions and in the absence of microbes and higher concentration of combined nitrogen (more than 1 mM) alfalfa develops some nodule-like structure, which are very similar to the morphology and tissue characteristics of the normal nodules (19). As a consequence, all those genes and functions, which are necessary for nodule development are encoded by the plant genetic material. However, the Nod factors produced by the compatible microosymbiont are responsible to what extend, and what type of nodules are formed by the plant.

Several genes, both bacterial and plant origin, are involved in the development and effective functioning of the nodules. Most of the genes in *S. Meliloti* and in other *Rhizobia* are knnown (5, 20, and personal information from Michael Göttfert, Technische Universität Dresden, Germany). According to Prof. Alfred Pühler, University of Bielelfeld, Germany the nucleotide sequence of the entire *S. meliloti* geome is known. However, the identification and the function of the plant genes involved in this biological process is still in its infancy. At present, biochemical and combined genetic and biochemical approaches are applied to study plant genes. Genes identified by biochemical methods are questionable as to whether they are involved in symbiotic nitrogen fixation. The nodule specific expression of many genes isolated by biochemical approaches was demonstrated nevertheless their real function was not uncovered (21, 22, 23), or it turned out that they are not essential to effective symbiotic nitrogen fixation (24).

Among others the *Medicago* EST programs will be useful to get acquainted with the nodule specific genes. These programs generated until now more than 100 000 partial EST sequences. These sequences are open for public on the Internet (**http://www.ncbi.nlm.nih.gov**). Among these sequences there are three partial cDNA sequences homologous to the NORK gene of *M. truncatula*. Form the mere sequence, however, it is impossible to predict the function of the gene, therefore more analysis is needed to reveal the function. The most powerful tool for the study of gene functions if mutant alleles of the genes are available. One can generate a mutant allele of a gene, or one can isolate a gene from a mutant allele. The first possibility (homologue recombination) is not available for plants. The later, however, is feasible through map based cloning or insertion mutagenesis (tranposons, T-DNA) based gene isolation procedures. According to mutant phenotype (T-DNA insertion) only one gene has been isolated form *Lotus japonicus*: this gene (*nin*) encoded a transcription factor (25). In this patent we describe for the first time the isolation of a gene by map based cloning that is the isolation of the NORK gene with the help of the *Medicago sativa* mutant MN1008.

Classical genetic strategy leads to unambiguous results. The isolated gene is an essential component of the biological process in question if the gene affected by the mutation has a phenotype (unable to carry out certain functions or less active in a given function) and the gene isolated based on the phenotype. In the mutant individuals an essential function affected which is involved in the biological process studied. Appropriate methods are available by which the mutant gene ant ist wild type counterpart can be isolated. In order to acquire mutant individuals mutations can be induced by physical, chemical or biological means. Mutant genes, in which no insertions of known sequences are present, or the nature of the mutation is not known (like in the case of large deletions, inversions) can be isolated by the so-called map based cloning. The principal of the map based cloning is the following: first the phenotype is mapped genetically with molecular (DNA) markers, and then with the help of these markers chromosomal walking is performed and the gene is isolated form clones originated form large insert libraries (26).

Mutant plants impaired in symbiotic nitrogen fixation can be grouped into two categories: mutants in the first class are the Nod$^-$ mutants that is no nodules appear on their root system; mutants in the second class are the Fix$^-$ mutants which develop nodules or nodule like structures but the nodules are ineffective, that is no effective nitrogen fixation takes place. It is a characteristic phenomenon of both mutant types, that in the presence of the compatible bacteria and in the absence of combined nitrogen the symptoms of nitrogen deficiency develop on the mutants, that is yellow leaves and stunted growth. There are many symbiotic mutants available published in the literature. Among the Nod$^-$ mutants the most important ones are the *Medicago sativa* MN1008 mutant (27, 28), the *Medicago truncatula* B129, Tr25, Trv25, B85 mutants (29), the *Pisum sativum sym*8, *sym*10, *sym*19 mutants (30), the *Lotus japonicus sym*2 mutant (31), and the *Glycine max nod*49 and *nod*139 mutants (14).

The MnNC-1008 (NN) alfalfa (*Medicago sativa*) mutant (abbreviated as MN1008) is incapable to form nodules on its root system in the presence of its compatible microsymbiont (*Sinorhizobium meliloti*) (27, 28). Further studies showed that there was no sign of any response detectable by microscopy, which was characteristic to the wild type plant early nodulation response. There was no root hair deformation and cortical cell division (32), in addition to the lack of the specific calcium spiking response (special calcium oscillation inside the cells) (33). According to unpublished results, there was no root hair curling or cortical cell division observed in the mutant MN1008 (unlike in the wild type plant) upon addition the specific *S. meliloti* Nod factor. Mutant MN1008, on the other hand, was able to develop nodules, the so-called spontaneous nodules, in the absence of microbes (see above). This phenomenon reflects that the plant nodulation program was intact. From the above results the most plausible conclusion was that the alfalfa mutant MN-1008 had a defect in the perception of the Nod factor, that is the assumed Nod factor receptor gene suffered mutation, or one of the member in the early signal transduction chain.

DISCLOSURE OF INVENTION

In agreement with the patent the knowledge and use of the DNA sequence, the described technology, specifically the isolation of the Nod factor receptor gene, or individual genes in the signal transduction has a great importance in the agriculture. The transfer of these genes into plants which are unable to fix nitrogen but have great agricultural importance, plants like rice, corn, wheat, barley, rye and so on, may ensure the possibility, that in the presence of appropriate Nod factors produced by *Rhizobium* bacteria the transgenic plants carrying and expressing Nod factor receptor genes and genes of the member of the signal transduction chain might be able to perform the first biological steps leading to nodule formation. Cloning and transfer other nodule specific genes might lead to the formation of normal symbiotic nodule and nitrogen fixation.

In accordance with the above, the invention relates to the *Medicago sativa* DNA sequence encoding the NORK polypeptide as well as to the protein determined by the mentioned DNA sequence. In addition the invention features conserved DNA regions, primers, DNA probes, polypeptides and corresponding antibodies characteristic to the NORK gene family, as well as to methods and their utilization to isolate additional NORK genes from different legumes or other plants, and to use those genes for producing transgenic plants.

In another aspect, the invention features a method of cloning a DNA sequence encoding a function involved in one of the steps of symbiotic nitrogen fixation, as well as to methods of producing transgenic plant cells and cloned entire plants wherein the biosynthesis of the NORK polypeptide expresses.

The experiments described in this invention relates to the identification and isolation of genetic determinants (genes) involved in the determination and development of symbiotic nodules of alfalfa (*Medicago sativa*) and in the perception and/or signal transduction of the Nod factor originating from the bacteria, with the help of the MN-1008 mutant incapable for Nod factor triggered

nodule development, on such a way, that the mutant phenotype is genetically mapped with the use of molecular markers, followed by the isolation of overlapping BAC clones using the tightly linked markers and by recombinant DNA techniques, then sequencing the genes in the overlapping BAC clones, and finally sequencing the mutant alleles responsible for the mutant phenotype. Applying the above procedure the following nucleotide sequence was identified:

CCATATTTTAACAATATTCTTTCTTCTACAAGGGTATAACTTTTATACAAGTTCACTATA
TTATAGGATTGATCAAGGTTCATTTTTTCTTTCTTTGAAAAATCTCTAAGGGGTGTGGTT
TCCAAGGCAGAAAATGAAATAGAATGCAGAAGAATTTGTATGGTACTATAAAGGGAAGAT
GAAAAGTTAGTTAGCATGGATTCAAGTTTGATAACCCTTTGGGGTAAAATCTCTTTCAGA
TTATGATGGAGCTACAAGTTATTAGGATATTTAGATTGGTTGTGGCATGTGTTCTTTGTT
TGTGTATATTTATCAGATCAGCTTCTTCTGCAACTAAAGGGTTTGAGAGCATATCATGTT
GTGCTGATTCCAATTACACAGATCCAAAAACAACCCTAACTTATACAACAGATCACATCT
GGTTCTCTGATAAAAGAAGTTGCAGACCAATACCCGAAATTTTGTTTAGCCACAGAAGCA
ATAAAAATGTTCGAATATTTGAAATAGATGAAGGAAAGAGATGTTATACTTTGCCAACAA
TTAAGGATCAAGTATATTTGATAAGGGGTGTATTTCCCTTTGATAGTTTAAATTCTTCGT
TTTATGTTTATATCGGGGTAACAGAACTAGGTGAATTAAGATCGTCTAGACTCGAGGACT
TGGAAATCGAGGGAGTTTTTAGAGCCACCAAAGACTATATTGATTTCTGCTTATTGAAGG
AAGATGTCAATCCCTTCATTTCTCAGATTGAATTGAGGCCATTACCTGAAGAATACCTAC
ATGGTTTCGCTACTAGTGTTTTAAAACTGATAAGCAGAAATAATCTTGGTGACACAAATG
ATGATATAAGGTTCCCAGATGACCAAAATGATAGAATCTGGAAACGGAAAGCAACTTCAA
CTCCATCATCTGCCCTTCCCCTGTCTTCCAATGTCAGCAATGTTGACCTCAAAGACAGTG
TCACACCTCCTCTACAAGTCCTACAAACAGCTCTTACTCACCCTGAGCGATTGGAGTTCG
TCCATGATGGCCTCGAGACCGATGATTATGAATACTCTGTGTTTCTCCACTTTCTTGAAC
TAAATGGCACTGTCAGAGCAGGACAAAGGGTGTTTGACATCTATCTAAACAATGAGATTA
AAAAGGAGAAGTTTGATGTTTTGGCTGGAGGGTCCAAGAACAGTTACACTGCCTTGAACA
TTTCAGCAAATGGATCACTCAATATAACCTTAGTCAAGGCATCTGGATCTGAGTTTGGAC
CCCTTTTGAATGCCTATGAAATCCTGCAGGCACGGTCGTGGATTGAAGAGACCAACCAAA
AAGATTTGGAACTTATTCAGAAGATGAGAGAAGAACTGCTGCTGCACAACCGAGAAAATG
AAGCATTGGAGAGTTGGAGTGGAGACCCTTGTATGATTTTCCCCTGGAAAGGAATAACAT
GTGATGATTCAACTGGTTCATCTATTATCACTATGCTGGATCTTTCTTCCAATAATCTCA
AGGGAGCAATTCCTTACTTTGTCACTAAGATGACCAATTTACAAATACTGAACCTGAGCC
ACAACCAGTTCGATTCGTTATTCCCCTCGTTTCCACCGTCCTCCTTGCTGATATCATTGG
ATCTGAGCTACAATGATCTTGATGGACGGCTTCCAGAATCCATTATCTCACTGCCACATT
TAAAATCATTATATTTTGGCTGCAATCCATATATGAAGGACGAAGATACAACAAAGTTGA
ACAGTTCACTAATCAATACAGATTATGGGAGATGCAAAGGAAAAAAACCAAAGTTTGGAC
AAGTATTCGTGATTGGAGCTATTACAAGGGGATCACTTTTGATTACTTTGGCTGTTGGAA
TTCTATTTTTTTGCCGTTATAGACACAAGTCAATTACTTTGGAAGGATTTGGTGGAAAGA

CCTACCCAATGGCAACAAATATAATCTTCTCTTTGCCAAGCAAAGACGATTTCTTCATAA
AGTCTGTATCAGTTAAACCATTCACTTTGGAGTATATAGAGCAGGCTACAGAACAGTACA
AAACTTTGATAGGTGAAGGAGGATTTGGTTCTGTTTACAGAGGCACTCTAGACGATGGTC
AAGAAGTGGCAGTGAAAGTGCGGTCATCCACATCAACTCAGGGAACCCNAGAATTTGATA
ATGAGCTAAACCTACTTTCAGCTATACAACATGAGAACCTGGTGCCTCTTCTGGGTTACT
GTAATGAGTATGATCAACAAATTCTCGTGTATCCATTCATGTCCAATGGCTCTTTGCTAG
ATAGACTATACGGGGAAGCATCAAAGAGAAAAATATTAGACTGGCCAACTAGACTCTCTA
TTGCTCTCGGTGCAGCTCGAGGTTTGGCATATCTTCACACATTTCCAGGACGTTCTGTAA
TACACAGGGACGTAAAATCGAGCAATATACTGCTGGATCAGAGCATGTGTGCTAAGGTTG
CAGATTTTGGTTTCTCAAAATACGCTCCTCAGGAAGGAGACAGTTATGTTTCCCTTGAAG
TAAGAGGAACTGCAGGGTATCTGGATCCTGAGTACTACAAAACCCAGCAATTATCTGAAA
AAAGTGATGTTTTCAGCTTTGGTGTGGTTCTACTTGAAATTGTAAGTGGACGGGAACCTC
TCAACATAAAGAGACCACGGATCGAGTGGAGCTTGGTTGAATGGGCTAAACCATACATAA
GAGCATCAAAGGTGGATGAAATTGTAGATCCTGGCATCAAGGGAGGATATCATGCAGAAG
CATTGTGGAGAGTTGTGGAAGTAGCACTGCAATGTCTAGAACCCTACTCAACATATAGGC
CATGCATGGTTGATATTGTCCGCGAGTTGGAGGATGCTCTCATTATTGAAAACAATGCAT
CTGAATACATGAAATCCATAGACAGCCTTGGAGGATCCAACCGCTACTCAATTGTTATGG
ACAAACGGGCGCTGCCTTCAACTACATCTACAGCAGAATCAACTATCACAACCCAAACCT
TGACACACCCTCAACCGAGATAGTAAATGGGTCGATGGAATTCTTTTGATTTGTTTTTTA
TCATTGCTTTAGTAATATCCCATTTTAAATGGTAAAGGAGAAAAATACTACTTTTGATTG
TATTTTCATCCACTCTATGTTTCTTGAAACTGAATCTCTCTTGCTCAGCCCCAGTTTTTA
TGGGTGAAAAGAATAATTTGGGTCAAATGCAAGTGAAACCATATGGTGCATAATTTGAAA
GCCATATTATATCATTTGCTAAGTCCAAAGTAAAAATTTCACAAACTAGTTAGATTGCGA
TTTAGTCTATACACACTTCAACAGAGCTATATACACTAT

The invention relates to the above genomic or synthetic nucleotide sequence molecules, to the amino acid sequence deduced from this DNA, to its deletion or mutant derivatives, to its recombinant forms, and to any other proteins having NORK specific biological activity. The invention relates to those oligonucleotides which are hybridizing to the above mentioned nucleotide sequence. In another aspect, the invention features those antibodies suitable for the detection of NORK polypeptides. In addition the invention relates to cells and transgenic plants as well transformed with DNA molecules protected by this invention.

The invention presents a procedure by which certain plants with Nod^- phenotype can be converted to Nod^+ phenotype.

To achieve the aim of the invention the MN-1008 mutant plant was grown. As opposed to the wild type plant, mutant MN-1008 respond (does not start nodulation consequently does not form nodules) neither to the compatible *Sinorhizobium meliloti* bacterium, nor to the Nod factor produced by the bacteria. It is supposed that the mutation in MN-1008 plant affected one of the genes involved in the signal perception and/or one of the early steps in the signal transduction pathway, it is likely the mutation affected the Nod factor receptor genes.

Under appropriate conditions, e.g. in the presence of sufficient nitrogen supply or in soil mutant MN1008 can grow and start to flourish. Mutant MN1008 was crossed with a nodulating wild type plant, the F1 generation and then by self-mating the F2 populations were established. In the F2 population (mapping or segregating population) the traits are segregating. From the individuals of the F2 population Nod^- and Nod^+ individuals were colleted (see Example 1.) and linked genetic markers were looked for (see Example 2.). With the help of the diploid mapping population the linked markers were mapped and it was concluded that all these markers mapped in the same region (see Example 3.). According to the map position of the diploid map, linked markers were selected from this region, which were mapped in the Nod^- segregating population (see Example 4.). It turned out that these markers were linked to the Nod^- phenotype, therefore mapped in the region where the Nod^- mutation mapped to, and two of these were tightly linked (see Example 4.).

With the help of the tightly linked markers so-called BAC clones were isolated from the BAC library of *M. truncatula,* followed by the ordering of the BAC clones into so-called contigs (see Example 5.). The so-called Nod contig was about a 500 kilobase-pairs (kb) long DNA region consisting of overlapping individual BAC clones. By the analysis of about 5000 F2 plants it turned out that in two individuals the recombination breakpoint situated within the Nod contig flanking both sides. This region was about 400 kb containing the Nod^- mutation (see Example 7.).

The BAC clones constituting the 400 kb contig were sub-cloned (see Example 6.), and the nucleotide sequence of the Nod region was determined, and the genes in this region were identified (see Example 8.). The Nod$^-$ mutant phenotype could be explained by mutation in some of the genes. Without limitation only two genes are mentioned as an example. One of these genes is an ABC transporter gene. Mutation in ABC transporter genes are responsible for the multidrog resistance phenotype (34) and for the human cystic fibrosis disease (35), therefore it is possible that the Nod factor uptake is mediated by ABC transporter proteins. The other gene is coding for a receptor kinase which was named as the NORK gene. The receptor kinase genes in plants are responsible for the perception and transduction of signal molecules (36, 37, 38). The lack of one of these functions could lead to Nod$^-$ phenotype. However, there are some more genes in the Nod region (see Fig. 7.) of which some are listed like the ODF gene, the MADS box gene, and the lectin genes, which could be responsible for the Nod$^-$ phenotype if they carry mutation alone or in any combination.

After the sequencing of the wild type allele of these genes, the mutant alleles were also sequenced on such a way, that gene specific primer pairs were designed, synthesized and used for the amplification of the appropriate DNA region of the MN-1008 mutant plant. No nucleotide changes were found in the ABC transporter, ODF, MADS box and lectin genes which could be responsible for the mutant phenotype. Some allelic variations were found, but these mutations could be neutral changes. On the other hand, however, an in frame stop codon mutation was found in the coding region of the NORK gene originated from the MN-1008 mutant (see Fig. 9) which terminated the protein synthesis before the naturally occurring termination. As a consequence the mutant would be shorter as compared to the wild type protein, and therefore the protein is inactive. According to the sequence analysis the mutation (the stop codon) occurred in the kinase domain in a region which is functionally active and highly conserved. With the help of the RT-PCR method and specific primers (see Fig. 9.) NORK specific cDNA fragments could be amplified. According to this result NORK specific receptor

kinase mRNA was synthesized consequently the NORK gene was transcribed in both wild type and mutant plants.

It is obvious for experts in molecular biology that the mutation in the NORK gene (the *nn1* mutation which generated an in frame stop codon) leads to loss of function because the active site needed for the kinase activity was missing (see Fig. 13). It is not excluded, however, that more mutation in the MN-1008 plant occurred (like in the ABC transporte gene, ODF gene, MADS box gene, and lectine gene), and the mutation leads to Nod$^-$ phenotype as well, consequently, these genes are protected by this invention too.

Abbreviations used in this inventions:

NORK:	NOD region specific Receptor Kinase gene.
nod genes:	Genes found in Rhizobium bacteria and which are responsible for the synthesis and excretion of the Nod factor.
Nod factor:	The lipo-chitooligosaccharide produced by *Rhizobium* bacteria which induces nodule formation on plant roots (see Fig. 1.).
Nod$^-$:	Phenotype of those plants lacking nodules
Nod+:	Phenotype of those plants with nodules
Diploid:	Somatic cells of the plants with double chromosome set
Teraploid:	Somatic cells of the plants with quadruplex chromosome set
RFLP marker:	Restriction Fragment Length Polymorphism, which is used as genetic marker. The lenght polymorphis is detected in most cases by DNA-DNA huybridization.
SHMT:	Serine-hydoxymethyl-transferase
BAC:	Bacterial Artificial Chromosome
BAC vector:	Low copy number cloning vector which can accomodate large (more than 100 kb) DNA fragments.

BAC clone:	Insert cloned into BAC vector, or both the vector and the insert together.
BAC library:	BAC clones representing the entire DNA content of an organism.
bp:	base pairs.
kb:	kilobase pairs.
allele:	gene variant.
primer:	short (5-50 bp long) single stranded oligonucleotide.
contig:	DNA region covered by overlapping clones
in frame:	according to the open reading frame
RT-PCR:	Reverse transcription PCR, that is amplification product of transcribed mRNA.
NAB:	one of the F2 family segregating the Nod$^-$ character
NBW:	the other F2 family segregating the Nod$^-$ character
RAPD:	Random Amplified Polymorphic DNA (46)

BRIEF DESCRIPTION OF DRAWINGS

Fig.1. Structure of the signal molecules (Nod factors) produced by *Rhizobium* bacteria. Nod factors produced by: A. *Sinorhizobium meliloti*; B. *Rhizobium leguminosarum* bv. *viciae*; C. *Bradyrhizobium japonicum*; D. *Azorhizobium caulinodans*; E. *Rhizobium tropici*; F. *Rhizobium* sp. NGR234.

Fig. 2. F2 alfalfa populations segregating the Nod$^-$ phenotype. The *nn1*/NN1 alleles are highlighted.

A. Crossing of the parents; highlighting linkage group 5 with the *nn1* és NN_1 alleles

B. The *nn1* and NN_1 alleles inherited by the F1 NAB plant.

C. Segregating *nn1*/NN_1 alleles in the F2 population originating from self mating of the F1 NAB plant.

D. Generating F3 homozygous individulas from the self mating of the appropriate F2 individuals.

Fig.3. Genetic mapping of the *nn1* gene in the tetraploid and diploid populations of *Medicago sativa*. Markers linked to the Nod$^-$ phenotype were screened by the Bulked Segregant Analysis with the help of the Nod$^-$ F2 NAB plants and Operon Primers. The position of the selected RAPD markers were then determined on the diploid alfalfa map. Finally RFLP markers linked to the RAPD markers were picked and mapped in the tetraploid population in relation to the *nn1* gene.

Fig. 4. Functional and restriction map of vector pBeloBac11. The cloning site (H = *Hin*dIII) sits in the *lacZ* gene. B = *Bam*HI; CM = chloramphenicol, see ref (52).

Fig.5. BAC clones in the Nod contig. Primary clones isolated by the help of the Q5E and SHMT markers are highlighted by green. Blue colors represent secondary clones isolated with the help of the end sequences of the primary clones. „b" and „j" represent the left and right side of the inserts, respectively.

Fig. 6. Determination of the precise position of the *nn1* gene with the help of two close recombination events. The precise localization of the *nn1* gene was established with the help of two DNA originating from individuals carrying recombination events in the Nod region, as well as with the help of two DNAs originating from opposite homozygous individuals. Sub-clone G34P44 originating from BAC 67A11 was used as hybridization probe for filter containing EcoRV digested DNA from NAB4156 (recombinant), NAB814 (homozygous), NAB4443 (recombinant), and NAB2161 (homozygous) individuals. Nod$^+$ allele (highlighted by + arrow) could be detected for the two Nod$^+$ plants (NAB4156 and NAB 814), while Nod$^-$ allele for the two Nod$^-$ plants (NAB4443 and NAB2161). Sub-clone G33P135 has been used to identify a Nod$^-$ allele for NAB4156. Sub-clone G3P126 identified Nod$^-$ allele for plant NAB4443 after DraI digestion. Sub-clone G18P4 on the other hand detected a Nod$^+$ allele for this plant after HindIII digestion. Since the two plants displaying recombination were Nod$^-$ the *nn1* gene can be limited between these two recombination points.

Fig.7. Genes and there orientation found in the Nod region. The arrows show the orientation of the genes in the Nod region. The lengths of the arrows are out of scale. pNORK_Nhe5 is a pUC19 based recombinant plasmid carrying the

entire NORK gene on a 8.5 kb long NheI fragment. The name of the genes are given after the abbreviations. The accession number of that gene is given which showed the highes BLST score in the NCBI databank. Finally the e value is given, which is a generally accepted value representing the level of homology.

Fig. 8. Nucleotide sequence of the 8563 bp long DNA region containing the entire NORK gene. The transcription start site (ATG) is at position 1153 bp (left to right) form the NheI site (GCTAGC). The stop codon is at position 663 bp from (right to left) the last nucleotide of the second NheI site. There are 15 exons and 14 introns in the gene. The position of the exons and introns are highlighted in Table 6. (There are 60 nucleotides in one row).

Fig. 9. List of the oligonucleotide primers used for the amplification of both genomic and cDNA sequences.

A. Name, sequence, length, and start points of the primers.

B. Schematic representation of the NORK gene with the position of the primers.

Fig. 10. cDNA sequence of the NORK gene from *Medicago truncatula* A17 (There are 60 nucleotides in one row).

Fig.11. Alignment of the genomic (see Fig 8.) and cDNA (see Fig. 9.) sequence of the NORK gene from *Medicago truncatula* A17. The genomic sequence (BAC A17) of the NORK gene identified in *Medicago truncatula* A17 was aligned to the cDNA sequence (cDNS A17). For the genomic sequences corresponding to the BAC clones: intron sequences are highlighted by red, the rest are black (Promoter region, exons, 5' and 3' untranslated regions). cDNA sequences are highlighted by blue. Nucleotide sequences corresponding to the first two (GT) and last two (AG) nucleotides in the introns are double underlined. Start codon (ATG) is green, stop codon (TAG) is purple. NheI sites at the beginning and at the end of the sequences are highlighted by orange.

Fig. 12. Deduced amino acid sequence from the NORK cDNA of *M. truncatula* A17 plant. (* = stop codon).

Fig.13. Functional representation of the NORK protein by highlighting the characteristic domains.

A. The NORK protein is 925 aa long. The different structural/functional domains are highlighted by different colors.

B. More detailed description of the functional domains found in the NORK protein.

Fig.14. cDNA sequence of the NORK gene for the two Nod$^-$ alleles 1 and 2, in plants (NAB1241/6 and NAB701/28) of *Medicago sativa*

Fig. 15. cDNA sequence of the NORK gene for one of the Nod$^+$ alleles (allele 6) in plant (NAB615/28) of *Medicago sativa.*

Fig. 16. Deduced amino acid sequence of the cDNA for the NORK gene from Nod$^-$ plants (NAB1241/6 és NAB701/28) of *M. sativa*. (* = stop codon).

Fig.17. Deduced amino acid sequence of the cDNA for the NORK gene from Nod$^+$ plants (NAB615/28) of *M. sativa*. (* = stop codon).

Fig. 18. Alignment of the cDNA sequences of the NORK genes originating from *Medicago truncatula*, *Medicago sativa*, *Vicia villosa* and *Pisum sativum. Medicago* alleles are from *Medicago truncatula* A17 (MtA17), *M. sativa* Nod$^-$ allele (Ms1N$^-$) and Nod$^+$ allele (Ms6N+), as well as from *Vicia villosa* (Vv) and *Pisum sativum* (Ps). *M. sativa* Nod$^-$ alleles 1 and 2 were identical. Coding regions are grouped in three nucleotide groups according to the genetic code. The position of the introns are highlighted by light purple (i1-i14). Nucleotides are highlighted by red if they are different from the MtA17 sequence. Mutations creating stop codons are shown by arrows. At least two alles are in *V. villosa*. Ambiguous nucleotied are higlighted as: M=A or C; R=A or G; W=A or T; S=C or G; Y=C or T and K=G or T.

Fig. 19. Alignment of the deduced amino acid sequence of the NORK gene from *M. truncatula* A17, *M. sativa* allele 6 and allele 1 as well as from *Vicia villosa* and *Pisum sativum*. *Medicago* alleles are from *Medicago truncatula* A17 (MtA17), *M. sativa* Nod^- allele (Ms1N^-) and Nod^+ allele (Ms6N+), as well as from *Vicia villosa* (Vv) and *Pisum sativum* (Ps). *M. sativa* Nod^- alleles 1 and 2 were identical. Amino acid differed form the MtA17 sequence are highlighted by red. At least two alles are in *V. villosa*. The corresponding amino acids are separated by /. Stop codons are highlighted by red. (There are 60 aa in one row).

Fig. 20. RFLP pattern of the NORK gene for the individuals from the diploid mapping population to demonstrate the single copy nature of the NORK gene. Hybridization pattern of the of the parents (*M. sativa* ssp. *coerulea* w2 és *M. sativa* ssp. *quasifalcata* k93), the F1/1 and individuals of the F2 generation on a filter containing DraI digested total DNA and hybridized by NORK specific probe (see Example 10.). The DNA used for probe containes two DraI sites, which are probably present in the NORK gene of the diploid species. As a consequence there are at least three hybridizing bands. (Other DraI sites are not excluded in the intron sequences of the genomic DNA). The 212 bp long DraI fragment probably did not transfer to the filter. The 10.5 kb fragments were not polymorphic, as contrast to the small one which is polymorphic. According to the segregation of this fragment there is only one copy of the NORK gene in the diploid alfalfa. Mcw2=*Medicago sativa* ssp.*coerulea* w2; MqK93=*Medicago sativa* ssp. *quasifalcata* k93

Fig. 21. RFLP hybridization of the NORK gene from tetraploid alfalfa. Hybridization pattern of the Nod^-/Nod^+ M. sativa individulas from the segregating population. Total DNA was digested with HindIII, the probe contained only the extracellular part of the cDNA. On the autoradiogram, there is only one strong hybridization band for four individuals, and there are two bands for one individual. Plant NAB814 carries only alleles 5, and 6, respectively. The rest of the plants carry only allele 1 and 2 between the recombination sites (see Example 7.). Allele 5 and 6 can be distinguished according to the size of the two hybridization

fragment in NAB814. On the other hand, allele 1 and 2 are indistinguishable (only one hybridizing band). This hybridization pattern indicates that there is only one copy of the NORK gene in the tetraploid alfalfa as well.

Fig.22. Hybridization pattern of the *M. truncatula* total DNA to the NORK gene. Hybridization pattern of *M. truncatula* total DNA digested by EcoRI and EcoRV enzymes. The probe ("NORK specific") contained only the extracellular part of the cDNA. Hybridization bands represent sequences homologous to the NORK gene.

Fig.23. Hybridization pattern of total DNA from different legumes (*Sesbania*, *Cassia, Trifolium* és *Desmodium*) to the NORK gene. The hybridization pattern is shown on filters containing total DNA digested by *Eco*RI, *Eco*RV, *Dra*I and *Hin*dIII enzymes. The probe ("NORK specific") contained only the extracellular part of the cDNA (see Example 10.). Hybridization bands represent sequences homologous to the NORK gene. S. r. = *Sesbania rostrata*; C. e. = *Cassia emerginata*; T. p. = *Trifolium pratense*; D. sp. = *Desmodium* species.

Fig 24. Hybridization pattern of total DNA from different legumes (*Vicia*, *Trifolium* és *Melilotus*) to the NORK gene. The hybridization pattern is shown on filters containing total DNA digested by *Eco*RI, *Eco*RV, *Dra*I and *Hin*dIII enzymes. The probe ("NORK specific") contained only the extracellular part of the cDNA (see Example 10.). Hybridization bands represent sequences homologous to the NORK gene. V. s. = *Vicia sativa*; M. a. = *Melilotus alba*; T. p. = *Trifolium pratense*; T. i. = *Trifolium incarnatum.*

Fig 25. Hybridization pattern of total DNA from different legumes (*Vigna* és *Macroptilium*) to the NORK gene. The hybridization pattern is shown on filters containing total DNA digested by *Eco*RI, *Eco*RV, *Dra*I and *Hin*dIII enzymes. The probe ("NORK specific") contained only the extracellular part of the cDNA (see Example 10.). Hybridization bands represent sequences homologous to the NORK

gene. V. u. = *Vigna unguiculata*; M. a. = *Macroptilium atropurpureum*; V. r. = *Vigna radiata*

Fig 26. Hybridization pattern of total DNA from different legumes (*Pisum*, *Glycine*, *Lotus*) to the NORK gene. The hybridization pattern is shown on filters containing total DNA digested by *Eco*RI enzyme. The probe ("NORK specific") contained only the extracellular part of the cDNA (see Example 10.). Hybridization bands represent sequences homologous to the NORK gene.

Fig 27. Hybridization pattern of total DNA from different non-legumes (rice, tobacco, wheat and corn) to the NORK gene. The hybridization pattern is shown on filters containing total DNA digested by *Eco*RI enzyme. The probe ("NORK specific") contained only the extracellular part of the cDNA (see Example 10.). Hybridization bands represent sequences homologous to the NORK gene.

Fig.28. cDNA sequence of the NORK gene from *Pisum sativum* cv. Frisson plant. (There are 60 nucleotides in one row).

Fig. 29. cDNA sequence of the NORK gene from *Vicia villosa* S-1 plant. (There are 60 nucleotides in one row). The nucleotides represented by the two allele of *V. villosa* are highlighted as follows: M = A or C, R = A or G, W = A ro T , S = C vagy G, Y = C or T, K = G or T .

Fig.30. Deduced amino acid sequence of the cDNA for the NORK gene from *P. sativum* cv. Frisson plant. Stop codon (*) is highlighted by red. (There are 60 aa in one row).

Fig. 31. Deduced amino acid sequence of the cDNA for the NORK gene from *V. villosa* S-1 plant. At least two alles are in *V. villosa.* The corresponding amino acids are separated by /. Stop codon (*) is highlighted by red. (There are 60 aa in one row).

Fig. 32. Antigen-antibody reaction using diluted (1:1000) rabbit serum with cell protein extracts from 10 mg plant tissue. A. control tobacco plant (non-transformed). B. Transgenic plant carrying the MtA17 NORK gene. C. Nod^+ control alfalfa NAB814. D. Nod^- alfafa (MN-1008).

Fig. 33. Multiple alignment of the NSL proteins. Amino acid residues were coloured according to an 80 % consensus. Letters mark the following amino acid classes: h = hydrophobic (ACFGHIKLMRTVWZ) and l = their aliphatic subset (ILV) with turquoise shading; a = aromatic (FHWY) with green shading; + = positive (HKR) with red shading; p = polar (CNEHKNQRST) in red; t = "turn-like" (ACDEGHKNQRST) in green; s = small (ACDGNPSTV) in blue. White letters with dark violet background mark amino acids identical (conserved 100%) in all sequences. Amino acids present in 80% of the sequences are shown by white letter with pink background. Stars indicate the positions where the amino acids of the legume NORKE domain differ significantly from the consensus of other NSL sequences. Mt = *Medicago truncatula*, At = *Arabidopsis thaliana*, Pv = *Phaseolus vulgaris*, Os = *Oryza sativa*, Gm = *Glycine max*, Gos = *Gossypum*, Zm = *Zea mays*, TC = tentative consensus sequences constructed from EST sequences by TIGR (www.tigr.org).

Table 1. Germination ability, viability and nodulation properties of the F2 seeds for the NAB and NBW families. F2 seeds were germinated on 1 % water-agar, after germination seedlings were transferred to tubes containing slant agar (Gibson). After one week plants were inoculated with *S. rhizobium* 41. After 6 weeks and 2 months nodulation was evaluated visually. For detailed description see Example 1.

Table 2. Genotyping the F2 NAB and NBW individuals for markers OPW8a, OPE8c, OPB13b, OPA6a and for nodulation. Total DNA from the highlighted NAB (15 Nod^- ; 15 Nod+) and NBW (8 Nod^-; 22 Nod+) individuals were amplified by OPW8, OPE8, OPB13, OPA6 Operon Primers as described in Example 2. During

the amplification reaction fragments (RAPD fragments) corresponding to markers OPW8a, OPE8c, OPB13b and OPA6a either appear (coded by number 5) or not (coded by number 3). Genotypes of the Nod^+ and Nod^- individuals are coded by number 5 or 3, respectively. Genotypes in the table are highlighted according to the concept of colormapping, colors without number are predicted genotypes (see ref. 39).

Table 3. Genotyping the OPW8a, OPE8c, OPB13b, OPA6a markers on the diploid mapping population. Genotyping of the maternal and parternal alleles corresponding to the hybridizing bands were done as follows: maternal homozygous = 1 (yellow); paternal homozygous = 3 (purple); hetrozygous = 2 (green); maternal dominant = 5 (light green); paternal dominant = 4 (blue); missing genotypes = 0 (grey). Colormapping was accordng to Table 3, prediction according to ref. 39. Colormaps show the U584B-LbIII region of linkage group 5 of the diploid *M. sativa* ssp. *coerulea* and *M. sativa* ssp. *quasifalcata* genetic map (40). 3A and 3B highlights the non-predicted and the predicted map, respectively.

Table 4. Genetic mapping of markers U71, U224, CG13, SHMT, Q5E és U492 on the tetraploid alfalfa population. Genotypes of the individuals plants were according to the size of the hybridizing band. If the band was similar to the size of the band from the Nod^- parent, the genotype code was 3 (purple). If the band was similar to the size of the band from the Nod^+ parent, the genotype code was 5 (light green). Genotypes of the Nod^+ and Nod^- individulas are coded by number 5 or 3, respectively. Genotypes in the table are highlighted according to the concept of colormapping, colors without number are predicted genotypes (see ref. 39).

Table 5. List of the sub-clones originating from BAC clones 28I12, 50E23, 2D11 és 67A11. The size of the insert in the sub-clones are also shown. For detailed description see Example 6. kb = kilobase pairs.

Table 6. Position of the exons and introns in the NORK gene according to Fig.9. Numbering starts from the first nucleotid of the NheI site (see Fig. 8.).

Table 7. Cloning of the NORK gene into plant cloning vectors. The description of the vectors used for plant trasnformation and the details of the cloning is given in Example 12.

Table 8. List of the Agrobacterium strains carrying the NORK gene. The construction of the *Agrobacterium rhizogenes* and *A. tumefaciens* strains used for the two different transformation protocols are given in Examples 12A and 12B, respectively.

Table 9. List of the *A. rhizogenes* strains used for transformation and the transformation frequency. Nod$^-$ plants were infected by *A. rhizogenes* strains as described in Example 12B. After emergence of the roots GUS staining was performed and the nodulation ability was tested. The second and the third row in the Table highlights the results obtained for the two transformation methods (combined). In brackets, the results from the second experiment are shown.

Table 10. Summary of the transformation experiments with *Medicago sativa* and *Agrobacterium tumefaciens*.

Table 11. Summary of the transformation experiments with *Nicotiana tabcum, Vicia villosa* and *Agrobacterium tumefaciens.*

BEST MODE OF CARRYING OUT THE INVENTION

Hereinafter the invention is demonstrated by examples. These examples do not restrict the protected domain of the invention they are only for demonstration purposes.

Example 1

Genetic crosses and the analysis of F2 progeny of the NAB and NBW families

The tetraploid (2n=4x=32) *Medicago sativa* MN-1008 mutant line (28) has been provided us by Prof. D. Barnes (University of Minnesota, MN, USA) (primarily called MnNC-1008 (NN), but generally abbreviated as MN-1008), is unable to form symbiotic nodules in the presence of compatible bacteria. The mutant plant emerged spontaneously by means of crosses between cultivated alfalfa populations and subsequent self-matings (27). One individual (referred in laboratory notebook as MN-1008/17) of the purple flowered MN-1008 mutant alfalfa line has been used as maternal parent in genetic crosses. The paternal parent was one individual of the *M. sativa* cv. Nagyszénási (referred as MsNa/5) alfalfa population. Since the self-pollinating efficiency of the MN-1008/17 maternal parent was between 10-20 %, its flowers had to be sterilized (emasculated). Prior to crosses the so-called banner petals of the matured flowers of the maternal parents were trimmed by scissors and followed by splitting open of the flowers by squeezing them to avoid the anthers to hit the stigma. Then the flowers were immersed in 51 % ethanol for 3-4 sec and then washed in water. After the flowers dried the stigma was covered with the pollens from the male parent. The efficiency of the ethanol treatment was checked in control experiments: the self-pollinating efficiency of the treated flowers in the absence of foreign pollen was about 0.5 %.

The F1 progeny seeds coming from the above described cross were vernalized at 5-8 °C for two weeks, then germinated and planted out first into plastic pots with diameter 8 cm, and after the plants gained strength into pots with diameter 25 cm containing flower-soil termed NT. Plants are maintained to bloom in greenhouse at 22-25 °C with photoperiod of 16 hours lighting and 8 hours dark. The flowers were self-pollinated one by one manually by squeezing the base of the flowers (tripping). At this time the stamen knocked against the stigma and the pollens got into the stigma from the anthers. After self-pollination the F2 ovules appeared on the plants from which the matured seeds developed. The F2 generation were begun to nurse in symbiotic test to determine their symbiotic phenotype. The matured seeds were vernalized and their surface were sterilized by rinsing them in 70% ethanol for 30 sec and treating with 0.1% $HgCl_2$ solution for 5 min followed with rinsing in sterile distilled water for five times. Then the

seeds were swelled in sterile water for 8 hours. After swelling, the seeds were placed in equable distances from each other on the surface of plates in Petri dishes containing 1% agar in distilled water. The dishes were incubated upside down in dark to prevent the emerging roots to grow into the agar medium. Next day the seedlings with roots ofabout 0.5 – 1.0 cm length were then placed on the surface of 1% agar slants with nitrogen-free Gibson medium (41) and the plants were grown at 22 - 25 °C with 16-h photoperiod (10 000 Lux). The seedlings were inoculated with *Sinorhizobium meliloti* 41 bacteria culture suspended in 1ml Gibson medium after 5-7 days of transfer.

The *S. meliloti* 41 bacteria culture were prepared for inoculate as follows. An inoculation loop of *S. meliloti* 41 strain (42, 43) stored at -80 °C were inoculated into test tube containing 5 ml YTB medium (44). The bacteria culture was rotated in test tube for 24 hours. The grown-up culture were inoculated into a 500 ml flask containing 100 ml YTB medium and the culture were grown up to stationer phase for about 24 hours. The concentration of the bacteria suspension grown-up to stationer phase was between 1-3 x 10^9 cells/ml. The bacteria were collected by centrifugation (in centrifuge Sorvall RC5 with GSA type angle rotor at 4 °C with 10 krpm for 10 min) under sterile conditions. The supernatant was discarded and the cells were resuspended in 100 ml Gibson medium (41). The concentrated bacteria culture was diluted in 100-fold in Gibson medium before inoculation. After dilution the concentration of the bacteria was 1-3 x 10^7 cells/ml. The nodulation phenotype of the plants was scored 6 weeks after inoculation, afterwards plants were potted into soil. Plants were removed gently out of the pots and were evaluated once more for the presence or absence of nodules 2 months later (secondary control of nodulation). The plants were planted back into pots and grown further. The flowers of the F2 individuals selected later by genetic analysis (determination of the genotypes for different molecular markers) were self-pollinated as described above to generate F3 seeds which were tested for symbiotic nodulation.

After the crosses 62 F1 seedlings were planted and grown up. Forty-four of these plants produced sufficient flowers and F2 seeds (at least 50-100 seeds/individuals) after self-pollination to test their viability and analyze the

segregation of symbiotic nodulation phenotype. Two F1 plants (NAB and NBW) producing the most vigorous F2 progeny in which the Nod$^-$ phenotype could be evaluated were selected for further experiments. The germinating, viability and nodule forming characters of NAB and NBW F2 seeds are listed in Table 1. The segregation ratio of the Nod$^-$ and Nod$^+$ characters was between 1:50 and 1:55 that is comparable to the segregation ratio of a single, recessive character in a tetraploid F2 segregation population (theoretical value is 1:35).

Composition of plates containing 1 % agar in distilled water:
10 g Bacto agar
1 liter distilled water
Sterilization for 30 min at 121 °C

Composition of flower-soil termed NT:
1 part Florina flower-soil (commercially available)
1 part peat (commercially available)
1 part sand from river Maros (commercially available)

Composition of YTB medium:
1% Bacto Tryptone
0.1% Yeast extract
0.5% NaCl
1 mM $MgSO_4$
1 mM $CaCl_2$

Example 2

Identification of linked RAPD markers to the *nn$_1$* gene of the Nod$^-$ MN-1008 mutant plant using the NAB and NBW F2 families

The identification of tightly linked markers to the *nn$_1$* gene, that is to the Nod$^-$ character was performed with the so-called Bulked Segregant Analysis method

(45). To perform the screen five Nod$^-$ and five Nod$^+$ plants were selected from the NAB and NBW families, respectively (Nod$^-$ NBW plants: NBW6, NBW20, NBW37, NBW162, NBW790; Nod$^+$ NBW plants: NBW9, NBW23, NBW74, NBW440, NBW718; Nod$^-$ NAB plants: NAB65, NAB267, NAB637, NAB701, NAB908; Nod$^+$ NAB plants: NAB26, NAB82, NAB139, NAB421, NAB814). The analysis of two F2 families enhanced the possibility to identify more linked markers. Since alfalfa is an outcrossing plant, the genome is heterozygous carrying different alleles for the genes. Analysing the inheritance of the alleles in a tetraploid population (as shown in Figure 2.) demonstrated that NAB F1 individual contained two Nod$^-$ alleles (indicated as 1 and 2) and two Nod$^+$ alleles (indicated as 5 and 6), while it did not carry the two other Nod$^+$ alleles (indicated as 7 and 8). As it turned out later, the analysis of the NBW family allowed us to detect polymorphism of these Nod$^+$ alleles (indicated as 7 and 8) and to identify additional linked molecular markers.

Total DNA was isolated from young leaves of the Nod$^-$ and Nod$^+$ plants using the QIAGEN Plant DNA Isolation Kit (DNeasy Plant Mini Kit; QIAGEN Inc. – USA; 28159 Avenue Stanford; Valencia CA 91355) according to the supplier's instructions. The concentration of the DNA samples were determined by spectrophotometer (OD_{260}= optical density measured at 260 nm) (1 OD_{260} = 50 μg/ml DNS). The DNA samples were diluted to a concentration of 5 ng/μl, and the DNA samples of five Nod$^-$ and Nod$^+$ plants were mixed, to produce the Nod$^-$ and Nod$^+$ homozygous groups. The mixed DNA samples of the homozygous groups were used as templates in polymerase chain reactions (PCR): 12.8 μl sterile distilled water, 5 μl DNS, 2.5 μl 25 mM $MgCl_2$, 2.5 μl 10 x Taq polymerase buffer (Zenon Kft, H-6720. Szeged, Berzsenyi u. 3.), 2 μl Operon Primer (2.5 pmol/μl), and 0.2 μl Taq DNS polymerase (5 U/μl) (Zenon) were pipetted into PCR tubes of 500 μl volume. The PCRs were carried out in 40 cycles with the following steps: 5 sec at 94 °C, 1 min at 37 °C and 1 min at 72 °C. After completion of the PCR amplification, 5 μl AGE (AGE = 20 % sucrose, 0.1 % bromophenol blue, 0.1 % xylene cyanol) stain was added to the samples and then the amplified products were separated in 2 % agarose gel (Sigma Low EEO) of 15 x 20 x 0.4 cm with 2 V/cm voltage for three hours in 1x TEA (40 mM Tris, pH 7.8, 50 mM EDTA, 50 mM acetic acid) buffer. After separation of the fragments, the gel were stained in 0.1 %

ethidium bromide solution and exposed to UV light to take pictures. The first 520 10-mer primers of Operon Primer set (Operon Technologies, Alameda, Ca, USA) with different nucleotide composition were tested to amplify of the DNA of the two homozygous bulks for both the NAB and NBW families. The PCR amplifications with these short primers resulted in fragment pattern typical for the given primer. The detected differences in the pattern (polymorphism) could be used as RAPD molecular markers (46). As a result of the tests of the primers of the Operon Primer set, several amplified fragments could be identified that were amplified on the bulk of the Nod^+ DNA but not on the bulk of the Nod^- DNA. Four out of them were analysed thoroughly: OPA6a (identified in NBW family), OPW8a (identified in NAB family), OPE8c és OPB13b (identified in both families). The sizes of the amplified products identified in the bulks of the Nod^+ plants were as follows: OPA6a, about 650 bp; OPW8a, about 300 bp; OPB13b, about 850 bp; OPE8c, about 480 bp. Afterwards the the individuals DNAs were re-tested in PCR to identify the genotypes. In addition other individuals from the families (15 NAB and 8 NBW Nod^- plants, as well as 15 NAB and 22 NBW Nod^+ plants) were tested for the same four RAPD markers. The result of the genotyping (thegenotypes) was displayed in color-map (39) in Table 2. The results presented in the table demonstrated unambiguously the linkage between the nn_1 gene and the four RAPD markers and determined the genetic order of the markers.

For fine mapping, the NAB family has been selected since it was the largest F2 population. The F2 individuals with diallelic configuration have been selected from this family to produce F3 individuals by self-mating (see Figure 2.). Individuals with homozygous genotypes for the different alleles were searched by determination of their genotypes. Three F3 plants were selected: the plant designated as NAB1241/6 that is Nod^- and contains the alleles labelled with no. 1 in homozygous configuration, the Nod^- plant designated as NAB701/28 containing the alleles labelled with no. 2 in homozygous configuration around the genomic region of the mutation and the Nod^+ plant designated as NAB615/28 carrying the alleles labelled with no. 6 in homozygous configuration. No individuals could be detected containing homozygous alleles labelled with no. 5 in this region.

Example 3

Genetic mapping of OPW8a, OPE8c, OPB13b és az OPA6a RAPD markers on the linkage map of diploid alfalfa (*M. sativa*)

In order to determine the map position of the nn_1 gene on the linkage map of alfalfa, the closely linked RAPD markers (markers OPW8a, OPE8c, OPB13b and OPA6a) were mapped on the genetic map of diploid alfalfa (40). To execute this experiment, the genotypes of the individuals of the diploid segregation population had to be determined. DNA fragments were amplified as described above using the OPW8, OPE8, OPB13 and OPA6 primers, respectively and the total DNA of NAB814 and NBW9 plants as template DNA. After separation, the DNA fragments corresponding to markers OPW8a, OPE8c, OPB13b and OPA6a were isolated from the agarose gels, purified by the QIAGEN Fragment Isolation Kit (QIAEX II Gel Extraction Kit; Cat.No. 200 51 QIAGEN Inc) and their concentration determined by spectrophotometer (determination of OD at 260 nm). Fifty ng of the fragments were labelled with deoxycitidine-5'-[alfa-^{32}P] triphosphate (Code: FP-205, Izotóp Intézet Kft., 1121 Budapest, Konkoly Thege Miklós út 29-33.) using the Ready-To-Go DNA labelling (-dCTP) Kit (cat. number: 27-9240-01; Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA) according to the suppliers' instructions. The HybondN$^+$ nylon filters (cat. number: RPN3003B, Amersham Pharmacia Biotech Inc, Little Chalfont, Buckinghamshire, England) carrying the restriction enzyme digested DNA of the individuals of the diploid mapping population were hybridized with the hybridization probes prepared as described above. The filters were prepared by the following method: total DNA was isolated of the individuals of the F2 mapping population (47) as described in Example 2. Aliquots of 20 μg DNA were digested with *Dra*I, *Eco*RI, *Eco*RV and *Hin*dIII restriction enzymes (Fermentas AB, Vilnius, Lithuania), respectively and the restriction fragments were separated in 0,8 %-os agarose gels as described in Example 2. The fragments were transferred onto HybondN$^+$ nylon membranes according to the suppliers' instructions and the filters were hybridized at 60 ^{o}C for 16 hours in CG buffer published by Church and Gilbert (48). After hybridization,

washing was carried out three times for 20 minutes each at the temperature of the hybridization in the following solutions: 1.) 0.1% SDS and 1xSSPE (49); 2.) 0.1% SDS and 0.5xSSPE; 3.) 0.1% SDS and 0.1xSSPE. Autoradiography of the filters was at –80 °C using the Sterling Diagnostic (Sterling Diagnostic Imaging Inc., Newark, DE 19714 USA) X-ray films or the filters were exposed in the exposure casette of the phosphorimager scanning instrument (Phosphorimager™ 445 SI; Molecular Dynamics Inc., Sunnyvale, CA, USA) and after the scanning of the screens the results were analysed by the ImageQuant computer software. The hybridizing fragments corresponding to the alleles of the OPW8a, OPE8c, OPB13b and OPA6a markers were identified as RFLP bands and the genotypes of the individuals were determined. The map position of the markers were determined by the colormapping procedure (39): all of the four markers were mapped in linkage group 5 (LG5) close to the U224 marker. The genotypes of the OPW8a, OPE8c, OPB13b and OPA6a markers of the individuals in the diploid mapping population and the results of the genetic mapping are presented in Table 3.

Example 4

Genetic mapping of the RFLP markers between U584B and LbIII markers in the tetraploid population segregating the *nn*$_1$ gene

To ensure that the map position identified in the diploid population with molecular markers linked in fact to the *nn*$_1$ gene (to the Nod$^-$ pnenotype) the map position of some markers mapped previously in this region (between the U584B and LbIII markers) were determined in the tetraploid alfalfa genetic map. The segregation of five RFLP markers (U71, U224, CG13, SHMT and U492) and a specific PCR marker (marked with Q5E) generated by cloning the amplified fragment (preparing the pQ5E palsmid) of the RAPD marker designated as OPQ5E were analyzed in the NAB population segregating the *nn*$_1$ gene.

Preparation of pQ5E plasmid: PCR amplification was performed using 50 ng total DNA of NAB814 plant (see the preparation of plant total DNA in Example 2) and OPQ5 primer as described in Example 2. The amplified products were separated in agarose gel (see Example 2) and the 1750 bp-long fragment corresponding to the Q5E marker were excised, reisolated from agarose gel and purified as described in Example 3. After adding 5.5 μl 10x Klenow buffer to the 50 μl eluated DNA solution, fragments were treated with 0.5 μl (5 unit) Klenow fragment of the DNA polymares I according to the suppliers' (Fermentas AB) instructions. Then 4 μl (1 μg) *Hin*cII-digested [prepared according to suppliers' (Fermentas AB) instructions], CIP(Calf Intestine Alkaline Phosphatase)-treated (dephosphorilation) and purified pUC19 plasmid (49), 7 μl 10x ligase buffer and 3 μl (15 U) T4 DNA Ligase enzyme (Fermentas AB) were added to the fragments. The ligation was performed at 14 °C for 24 hours. After this the ligation was transformed into 250 μl E. coli DH5α kompetent cells (Amersham Pharmacia Biotech AB, Uppsala, Sweden) using the $CaCl_2$ procedure (50). After induction of the bacterial gene which resulted in resistance against ampicillin (phenotypical expression), the bacteria were plated on LB plates containing ampicillin (100 μg/ml) and incubated at 37 °C for 16 hours. Plasmid DNA was isolated from the resistant colonies by QIAGEN plasmid isolation kit (QIAGEN Plasmid Mini Kit; Cat.

No. 12123 QIAGEN Inc.). One clone was selected by PCR amplification from the clones carrying the 1750 bp-long amplified product: BRC1464 = E. coli DH5α (pQ5E). The sequence of the insert was determined and specific primers were designed based on the sequence:

Q5EU1: 5'-CGGCGTCTTGCTAAAGGAGA-3';

Q5ED1: 5'-TATTCGACTCATCATGGTTA-3';

Q5EU2: 5'-GTCAAATATCGATTCGTGAT-3';

Q5EU3: 5'-CTAAGAAAGGCTTTGGTTGG-3';

Q5ED3: 5'-TCACGAATCGATATTTG ACA-3').

Using the specific primers in PCR amplification, the dominant OPQ5E marker was transformed into codominant Q5E marker and its more precise map position was determined in relation to the other markers.

The map position of markers U71, U224, CG13, Q5E és U492 have been published earlier (40). The SHMT marker (51) mapped between markers Q5E and U224 on the genetic map of diploid *M. sativa* (Kaló et al. manuscript in preparation). Markers U71, U224, CG13, SHMT and U492 were mapped as RFLP markers using the F2 NAB individuals, while the marker Q5E was mapped by specific PCR (94°C 30sec, 60 °C 1 min, 72 °C 1 min in 40 cycles) as well as by RFLP in the tetraploid population. The inserts of the clones (47, 51) corresponding to RFLP markers and pQ5E clone were reisolated, labelled, hybridized, washed and autoradiographed according to the method described in Example 3. The filters were selected for hybridization containing the *Dra*I, *Eco*RI, *Eco*RV and *Hin*dIII-digested total DNA of the selected Nod^- és Nod^+ NAB individuals. The genotypes of the individuals were determined for markers U71, U224, CG13, SHMT, Q5E and U492 by the hybridization signals corresponding to the different alleles. The order of the genes shown in Table 4 was determined by the colormap procedure. The summary of the genetic mapping of the nn_1 gene displaying the steps from the identification of the linked markers to the confirmation of the map position in the tetraploid population is presented on Figure 3. Analyzing the mapping data, it turned out that the markers SHMT and Q5E were the most tightly linked markers to the nn_1 gene. No recombination could be detected in the individuals of the

analyzed population between markers SHMT and nn_1. One recombination could be detected between markers nn_1 and Q5E in the analyzed individuals.

Example 5

Identification of overlapping BAC clones, constructing the contig

The primary BAC clones (67A11, 2D11 és 20K10) were isolated by two molecular markers (SHMT and Q5E, see Figure 3.) most tightly linked to nn_1 mutation in the following way: the Q5E fragment was isolated from plasmid pQ5E, while the SHMT fragment was excised from the plasmid containing the cDNA of the gene with *Kpn*I restriction enzyme (51). The fragments were reisolated after gel electrophoresis, labelled and the filters were hybridized, washed and autoradiographed as described in Example 3. The filters contained more than 30 000 clones of the BAC library constructed from the total DNA of *Medicago truncatula* a close relative of *Medicago sativa*, (53). The BAC clones in the library contained about 100 kb inserts of the total DNA of *M. truncatula* . Cloning was done by *Hin*dIII digestion of the total DNA and cloned into the *Hin*dIII restriction sites of the pBeloBACII vector (52) (Figure 4.). The filters and clones of the BAC library are available in a public collection (Clemson University Genome Institute,.100 Jordan Hall, Clemson 29634-5727, South Carolina, USA).

Two primary BAC clones (clones 67A11 and 2D11) was identified by hybridization with SHMT fragment, and one primary BAC clone (20K10) with the Q5E fragment. An inoculation loop of the BAC clones 67A11, 2D11 and 20K10 stored in the clone collection at -80 °C were taken from the stock and inoculated into 500 ml flask containing 100 ml LB media and chloramphenicol in 15 μg/ml concentration, respectively. The clones were grown to stationer phase at 37 °C in a shaker (250 rpm). The plasmid DNA of BAC clones were purified by the QIAGEN Large Construct Kit according to the suppliers' instructions. The plasmid DNA of the three clones were subjected to single *Eco*RV and double *Eco*RV/*Not*I digestion and the fragments were separated in agarose gels. Based on the pattern of the single and double digestion, the *Eco*RV end-fragments of the clones could be selected. From these fragments the end fragment of the inserts could be detected by *Hin*dIII digestion. These end-fragments were used for chromosomal walking, that is to identify overlapping BAC clones. The *Hin*dIII end-fragments of the clone

67A11 were approximately 3300 és 800 bp, of the clone 2D11 were 3300 and 4000 bp and of the clone 20K10 were appr. 1080 és 1400 bp length, respectively. The six end-fragments were reisolated from agarose gel and repeated hybridization was performed to identify and isolate the secondary BAC clones (50E23, 64B5, 28I12, 6A5). Based on the restriction digestion pattern (*Hin*dIII, *Eco*RI, *Eco*RV) of the primary and secondary BAC clones, the degree of the overlap of the clones could be determined and the position of the clones to each other could be established. In this way the Nod contig could be constructed (see Figure 5.).

Example 6

Subcloning of the Nod contig

The result of the genetic mapping revealed that the SHMT marker could be the closest marker to the nn_1 gene therefore the chromosomal region around the SHMT was analyzed first thoroughly by delimiting the genomic region containing the nn_1 gene and by determination of the sequence of this region. In parallel experiments the subcloning of two BAC clones (67A11, 2D11) identified by SHMT and the subcloning of clones 50E23 és 28I12 overlapping with 2D11 clone were needed. The subcloning was performed in three ways. On one hand (A) the ends of the BAC inserts in the contig were cloned, on the other hand (B) the 1-2 kb length fragments of the clones 67A11, 2D11 and 50E23 generated by sonicating were cloned, so random subcloning was performed, and on the third hand (C) cloning was realized after restriction enzyme digestion.

Example 6A

Subcloning of the end-fragments of the BAC inserts

The end-fragments of the BAC clones were idenified as described in Example 5 and the *Hin*dIII fragments were cloned into pUC19 plasmid in the

following way: 4 μl (1 μg), *Hin*dIII-digested and dephosphorylated pUC19 plasmid (Fermentas AB), 14 μl 5x Ligase buffer, and 2 μl (10 unit) T4 DNA Ligase enzyme (Fermentas AB) were added to the DNA fragments purified with the QIAGEN DNA Isolation Kit (see Example 3) and eluated in 50 μl buffer. The ligation was executed at 14 °C for 24 hours. The ligation was transformed into 250 μl E. coli DH5α competent cells using the $CaCl_2$ procedure (see Example 4). The transformant colonies were checked by the restriction enzyme digestion of the plasmid DNA isolated with QIAGEN plasmid DNA Isolation Kit (see Example 3).

Example 6B

Random subcloning of the inserts of the 67A11, 50E23 and 2D11 BAC clones

20 μg DNA of the clones 67A11, 50E23 and 2D11 in 200 μl TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH7.5), respectively, were sonicated for 10 sec with the smallest head of the MSE Ultrasonic Disintegrator equipment. The fragmentated DNA was separated in agarose gel and the 1-2 kb length fragments were excised, reisolated and purified as described in Example 3. The DNA fragments generated in this way were ligated into *Hin*cII-digested (Fermentas AB) and dephosphorilated pUC19 plasmid as described in Example 4 and then they were transformed into *E. coli* DH5α competent cells (see Example 6A). Plasmid DNA were isolated from the transformant colonies with QIAGEN Plasmid Isolation Kit and their contents for inserts were checked by electrophoresis in 1 % agarose gels.

Example 6B

Subcloning of the 67A11, 2D11, 50E23 and 28I12 BAC clones after restriction enzyme digestion

The plasmid DNA of BAC clones 67A11, 2D11, 50E23 and 28I12 were purified as described in Example 5 and 1 μg purified plasmid DNA were digested

with *Nhe*I, *Xba*I, *Hin*2I, *Hin*6I, *Tai*I, *Taq*I, *Bam*HI, *Hin*dIII, *Eco*RI and *Sau*IIIA1 enzymes (Fermentas AB), respectively according to the suppliers' recommendation. The *Nhe*I and *Xba*I fragments were cloned into *Xba*I-digested (the *Nhe*I and *Xba*I result in compatible sticky ends) and dephosphorylated pUC19 vector, the *Hin*2I, *Hin*6I, *Tai*I, *Taq*I fragments were cloned into *Acc*I-digested and dephosphorilated pUC19 vector, the *Hin*dIII and *Eco*RI fragments were cloned into *Hin*dIII- and *Eco*RI-digested and dephosphorilated pUC19 vector and the *Bam*HI and *Sau*IIIA1 fragments were cloned into *Bam*HI-digested and dephosphorilated pUC19 vector. After digestion the restriction fragments were purified by the QIAGEN DNA Isolation Kit (see Example 3), ligated into the corresponding enzyme-digested (*Nhe*I, *Acc*I, *Hin*dIII, *Eco*RI and *Bam*HI) and dephosphorilated pUC19 plasmid and transformed as described in the Example 6A. The transformant colonies were checked by digestion with the corresponding restriction enzyme after plasmid DNA isolation as described above (Example 6A).

The recombinant clones and their insert sizes generated with subcloning are presented in Table 5.

Example 7

Determination of the map position of the nn_1 gene more precisely in the contig by identification of the closest recombination sites at both sides of the mutation

The generation of the subclones from the BAC clones overlapping the nn_1 mutation and the determination of the map position of the nn_1 gene more precisely by this subclones were done parallelly. The generated subclones were used as genetic markers to determine the recombination sites more precisely. After having the recombination sites more precisely it was obvious that the detailed sequence analysis for BAC 67A11 and 2D11was necessary.

Two Nod$^-$ plants (NAB4156 és NAB4443) carrying recombination close to the nn_1 mutation and two other plants that did not contain recombination close to the Nod region but they were opposite homozygotes (Nod$^+$ NAB814 and Nod$^-$

NAB2161) for the Nod character were used for genetic experiments. The clones G34P44 and G33P155 coming from BAC clone 67A11, the clone G3P126 coming from 2D11 and clone G18P4 arising from 28I12 BAC clone were converted to molecular markers. The inserts of the clones were excised with the proper restriction enzyme, the fragments were reisolated and used as hybridization probes to the filters containing the *Eco*RV-, *Hin*dIII- and *Dra*I-digested total DNA of above mentioned four plants. From the experimantal results (see Figure 6) it could be concluded that the position of the nn_1 gene was delimited on a genomic region (Nod region) by two recombination detected by fragment G34P44 located on BAC clone 67A11 and by fragment G18P4 located on BAC clone 28I12.

Example 8

Determination and analysis of the sequence of the Nod region

In order to determine the sequences of the subclones obtained as described in Example 6, the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit and the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Applied Biosystems; 850 Lincoln Centre Drive Foster City, CA94404 USA) was used according to the manufacturer's recommendation. The plasmid DNA for templates of the seqeuncing reaction were isolated by QIAGEN Plasmid Isolation Kit and their concentration were quantified using spectrofotometry (see Example 3.). The sequences of the amplified products labelled by flurorescent dyes were determined by ABI 373 and ABI 377 automated sequencer (Perkin Elmer Applied Biosystems; 850 Lincoln Centre Drive Foster City, CA94404 USA). Authenticity of the DNA sequences: the sequences were determined on both strand, in one direction the reactions were repeated 2-8 times, based on independent templates in ambiguous cases. The sequence data were stored in computer and their analyses were started with ordering them into overlapping sequences. The overlapping end-fragments of the BAC clones and of the subclones generated by restriction digestion of these BAC clones could help to assemble the sequences and to arrange the subclones generated by random

fragmentation or restriction enzyme digestion and the BAC clones to each other. By the help of the available sequences, homologue genes were searched in the NCBI (National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov/BLAST/) and the *Arabidopsis* (http://www.arabidopsis.org) databanks. Taking into consideration of the homology between the sequences, the common and general characters of the gene structures [consensus sequences as initiation and termination codons, open reading frame (ORF), consensus sequences characteristics of exons and introns such as GT-AG rule, the point of divergence, etc.] the starting- and end-point of the coding regions of the genes located in the Nod region were searched and the order and the orientation of the genes were determined. The results of this sequence analysis are presented schematically on Figure 7.

Example 9.

Detailed analysis of the NORK gene

The Nod$^-$ phenotype of the mutant plant can be explained above all with a mutation in the NORK (NOD region linked Receptor Kinase) gene coding for a receptor kinase, that is why the sequence analyis of the wild type NORK gene on BAC clone 67A11 was carried out first. The NORK gene is located on an NheI fragment of 8563 bp length and its nucleotide sequence - from the *Nhe*I site to the next *Nhe*I site - is shown in Figure 8. This nucleotide sequence identify the genomic sequence of the NORK gene from the *M. truncatula* A17 plant (the BAC library was constructed from this plant). To reveal the exact structure of the gene we have to know the exon/intron borders/positions which can be predicted from the genomic sequence based on the GT-AG rule and the branching point, however, it can be determined exactly only by knowing the cDNA sequence. For this purpose we isolated RNA from the roots of *M. truncatula* line A17, then we prepared cDNA from the mRNA by reverse transcription. cDNA samples were produced by PCR

amplification using NORK specific primers(see Figure 9.), then the amplified fragments were sequenced either directly or after cloning as desribed in Example 8.

Example 9A.

Construction and sequencing of NORK specific cDNA sequences

The seeds of *M. truncatula* A17 were germinated after sterilization and planted into sterile soil. From the roots of the two-three week old plants the cDNA representing the NORK mRNAs as well, was prepared as follows. The roots of the plants were pulverized/ground under liquid nitrogen, the powder was solubilized in RNA extraction buffer (8 M guanidinium chloride, 20 mM MES, 20mM EDTA, 50 mM β-mercaptoethanol), then purified by extraction with a mixture of phenol-chlorophorme-isoamil-alcohol (25:24:1). After centrifugation the supernatant was precipitated at -20 °C for 1 hour with 0.7 volume of 95% ethanol and 0.2 volume of 1 M acetic acid. After centrifugation the precipitated RNA was dissolved in DEPC (diethyl-pyrocarbonate)-treated distilled water. The cDNA was prepared from the RNA with the help of the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas AB). The different RNS samples were treated with RNase free DNase (Fermentas AB) and then the cDNAs were synthetized with the help of oligo-dT primer and reverse transcriptase enzyme (Fermentas AB) in the presence of RNase inhibitor (Fermentas AB) at 42 °C. Overlapping fragments of the NORK cDNA were amplified from the prepared cDNA with different combinations of the primers shown in Figure 9. The PCR conditions were as follows: 95 °C for 30 seconds, 55 °C for 1 minute, 72 °C for 1 minute, these steps were repeated 35-times. The specific NORK fragments amplified by RT-PCR from the cDNA were separated by agarose gel electrophoresis and purified with the help of the Fragment Isolation Kit of QIAGEN. The purified fragments were sequenced as

described in Example 8. either directly or after cloning them into HincII digested and phosphatase treated pUC19 vector. The full-length cDNA sequence could be constructed from the sequences of the overlapping cDNA fragments. The cDNA sequence of the NORK gene from the *M. truncatula* A17 plant is shown in Figure 10.

Example 9B.

The alignment and analyis of the genomic and cDNA sequences of the NORK gene.

The exact posotion of the exons and introns could be determined by aligning the genomic and the cDNA sequences of the NORK gene. The alignment of the NORK gene sequences from the *M. truncatula* A17 plant is shown in Figure 11., the detailes about the position of exons and introns are presented in Table 6. The proposed start codone of the NORK gene is the ATG start codone (coloured green) at the 1153. bp of the *Nhe*I fragment (pNORK_Nhe5), while the proposed stop codone is the TAG stop codone (coloured violet) at the 7898. bp. In the putative promoter region at the 5' end of the gene can be found the TATA-box (743-55, 769-85) és CCAAT-box (675-79, 688-91 bp) sequences characteristic for the region. At the 3' end of the gene there are several sequences which may serve as transcriptional termination signals. There is no difference between the two sequences (genomic and cDNA) which is in accordance with the facts that the genomic and the cDNA is originated from the same plant, and there is only one gene in the genome.

By comparing the sequences to the entries of databases we realized that in the case of *M. truncatula*, in the so-called EST (Expressed Sequence Tags) databases there are partial cDNA sequences homologous to the NORK gene. These sequences were obtained by random sequencing, i.e. certain laboratories

made sequencing reactions and single runs on several thousand cDNA clones of different libraries without any knowledge about their function. Such sequences from the *M. truncatula* plant can be found under accession numbers AW684661, AW685681 and BE203249. The appearance of the cDNA sequences in this form also support the expression of the NORK gene in the roots of legumes.

The activity of the NORK gene in the cells, i. e. it is expressed, is proven by the fact that we could detect NORK specific cDNA corresponding to the mRNA via RT-PCR. We presented as described in Example 14. that the NORK protein is synthetized from the mRNA. The amino acid sequence of the NORK protein can be deduced from the cDNA sequence. The amino acid sequence deduced from the longest Open Reading Frame (ORF) of the NORK cDNA is shown in Figure 12. The NORK protein consists of 924 amino acids, and it contains characteristic sequence motifs pointing to functions, which were revealed on the basis of similarity to consensus sequences in databases. The functional representation of the NORK protein and its characteristic sequence motifs are shown in Figure 13.

From the above data obtained in this way we concluded that one part of the molecule is located extracellularly (extracellular part) which is followed by an intracellular part via a transmembrane stretch. According to our knowledge the extracellular part contains a so-called LRR (Leucin Rich Repeats) region (according to the homologies: between amino acids 405-476) which may be involved in either the direct or the indirect recognition and in either the direct or the indirect binding of Nod factor. Based on the similarities, the proposed intracellular part has kinase activity. It is known for a specialist of molecular biology that the kinases participate in different signal transduction pathways, they initiate or transduce the biological signals by phosphorilating proteins. The NORK protein may phosphorilate its substrates within the cell and thus it initiates the signal transduction cascade leading the development of the nodule. Based on the structural analysis of the protein it is possible to suppose that the NORK protein is capable for autophosphorilation.

Example 9C.

The alignment of the cDNA sequence of *M. truncatula* A17 to those of the mutant (Nod^-) and the wild type (Nod^+) *M. sativa* plants.

With the help of primers designed to the NORK gene of *M. truncatula* (Figure 9.) genomic and cDNA sequences (Example 9A.) were amplified from F3 plants carrying in homozygous configuration the Nod^- alleles designated as number 1 and number 2 (NAB1241/6 and NAB701/28), as well as the Nod^+ allele designated as number 6 (NAB615/28). The nucleotide sequences of the amplified products were determined directly or after cloning as described in Examples 8. and 9A. The cDNA sequence of the Nod^- alleles designated as number 1 and number 2, as well as the Nod^+ allele designated as number 6 is shown in Figures 14. and 15., respectively, the amino acid sequences deduced from the cDNA sequences of the Nod^- alleles designated as number 1 and number 2, as well as the Nod^+ allele designated as number 6 can be seen in Figures 16. and 17., respectively. The sequence analysis revealed that the sequences of the NORK alleles designated as number 1 and number 2 are identical, that is why only one sequence is presented.

The alignment of the cDNA sequences of the Nod^- and Nod^+ alleles from *M. sativa* to the cDNA sequence of the *M. truncatula* NORK gene is shown in Figure 18. One can see from the comparison of the sequences that there are a number of differences between the sequences of *M. sativa* and that of *M. truncatula*, and a few differences - in most cases causing no amino acid changes - can be found between the wild type and mutant alleles of *M. sativa*. The most dramatic change in the structure of the Nod^- allele carrying the nn_1 mutation can be found at the 11. basepair in the 13. exon (E13) of the gene. The mutation in the triplet coding for tirosine resulted in a STOP codone in the sequence originated from the Nod^- plant. As a result of this mutation, the synthesis of the protein is terminated earlier, even

before the synthesis of the putative active site of the kinase, and it can be supposed that the produced shorter protein cannot fulfil its function in the signal transduction..

The alignment of the amino acid sequences of the NORK proteins of the *M. truncatula* A17 és a *M. sativa* plants is shown in Figure 19. The comparison of the sequences reveals that the sequences of *M. sativa* differ at a number of positions from that of *M. truncatula* A17. The amino acid differences between the Nod^+ alleles of *M. truncatula* A17 and *M. sativa* are probably the results of so-called neutral mutations which do not change the function. The comparison of the amino acid sequnces of the Nod^- and the Nod^+ NORK alleles reveals that the most dramatic change in the structure of the NORK protein in the *M. sativa* mutant MN-1008 is that the protein consists of only 709 amino acids because there is a STOP codon in place of the tyrosine codon in position 710 which causes the termination of protein synthesis (see above). There are more than one amino acid changes in the protein originated from the Nod^- allele compared to that of the Nod^+ allele which might result in the loss of function. It cannot be decided that in which order the mutations in the nn_1 gene arose and which one was the first that in homozygous configuration resulted in the fail of nodule formation, i.e. in Nod^- phenotype.

Example 10.

The number of NORK genes in the *Medicago* genome and its distribution in the plant kingdom

The genetic mapping is a suitable tool to determine the copy number of the NORK gene. For this purpose, using the putative extracellular part of the NORK gene (this part is specific, there is no homologous sequence in the databasis) as a hybridization probe, hybridization experiments were carried out as described in Example 3. with the filters carrying the DNA of the individuals from the diploid and

tetraploid mapping populations of *M. sativa*. The 1695 bp length probe, which is called as „NORK specific" probe, was prepared by PCR using primers Pr_RKU4 és Pr_RKD2X (Figure 9.) and a cDNA template originated from the *M. truncatula* A17 plant as described in Example 9A. The analysis of the autoradiograms showed that the segregating hybridizing fragments identify a single locus (Figures 20. and 21.), which means that there is only one copy of the NORK gene in the genome, i.e. the NORK gene is a single-copy gene.

The above described probe was used for the hybridization to the genomic DNA of different plants. With the help of the QIAGEN Plant DNA Isolation Kit genomic DNA was isolated as described in Example 2. from different legume plants: *M. truncatula* A17 és A20, *Pisum sativum* cv. Frisson, *Sesbania rostrata*, *Cassia emerginata*, *Desmodium* sp., *Vicia sativa*, *Melilotus alba*, *Trifolium pratense*, *Trifolium incarnatum*, *Vigna unguiculata*, *Macroptilium atropurpureum*, *Vigna radiata*, *Glycine max*, *Lotus corniculatus*, *Lotus japonicus* cv. Gifu, and from non-legume ones: *Nicotiana tabacum* cv. Small Havanna SR1, and rice. 15-20 μg of total DNA was digested by restriction enzymes *Eco*RI, *Eco*RV, *Dra*I or *Hin*dIII, the DNA fragments were separated by electrophoresis and bound to Hybond N+ filters (see. Example 3). The autoradiograms obtained from the hybridizations performed at 55 °C using the „NORK specific" probe (Example 10.) can be seen in Figures 22., 23., 24., 25., 26., 27. One can see on the autoradiograms that there is only one or two strongly hybridizing fragments in most legumes which means that in most of the legumes the NORK gene is a low copy number gene, only one or few genes are present in the genome. The autoradiograms obtained after the hybridization experiments with non-legume plants show that in these species the presence of the NORK gene cannot be detected under our hybridization conditions.

Example 11.

Determination of the NORK cDNA sequence from other legumes.

The nucleotide sequences of the NORK gene weres determined from a single individual of two other legume species, pea (*Pisum sativum*) and vetch (*Vicia villosa*). The sequencing of the NORK cDNA from *Pisum sativum* cv. Frisson and *Vicia villosa* S-1 plants was carried out using the primers designed for the NORK gene of *Medicago truncatula* (Figure 9.) as described in Example 9A. Both *Pisum sativum* cv. Frisson and *Vicia villosa* S-1 has Nod^+ phenotype, similarly to *Medicago truncatula*, diploid, autogamous plants, which means that the two alleles of their NORK gene has identical sequence. The nucleotide sequence of the amplified products was determined directly or after cloning as described in Examples 8. and 9A. Figures 28. and 29. show the NORK cDNA sequences of *Pisum sativum* cv. Frisson and *Vicia villosa* S-1, respectively, constructed from the sequences of overlapping fragments. Figures 30. and 31. show the amino acid sequences deduced from the cDNA sequences of these plants.

The cDNA sequences determined by us from *Pisum sativum* and *Vicia villosa* plants as well as the cDNA sequence from the *Lotus japonicus* Miyakojima MG-20 plant deposited in the NCBI database under the Accession number AV410167 proves that the NORK gene is transcribed in these plants as well, , i.e. it is active. It is noteworthy to mention that in *Vicia villosa* there are either two alleles or two copies of the NORK gene (see Figures 29. and 31.). It can be concluded as well that th e nucleotide sequence of the NORK gene in plants investigated by us is so homologous to that of *M. truncatula* that the primers designed for the NORK gene of *M. truncatula* could be used for the amplification of different parts of the NORK gene from alfalfa, pea and vetch.

Example 12.

Complementation of the Nod$^-$ mutant *M. sativa* MN-1008 plant with the NORK gene.

In order to prove that the identified mutation in the NORK gene is responsible for the Nod$^-$ mutant phenotype, the 8.5 kb *Nhe*I fragment carrying NORK gene (Table 5., Figures 7. and 8.) was cloned into plant transformation vectors (see Table 7.). The NORK gene was cloned in the so-called T-DNA part of the *Agrobacterium*-based plant transformation vectors. *Agrobacterium* species that entered the tissues of plants transfer the T-DNA from their plasmid into the plant cells (T-DNA transfer), which is followed by the integration of T-DNA (54). The random integration of T-DNA into different regions of the plant genome makes possible the stable maintenance and inheritance, as well as the expression of the genes of the T-DNA. There are two basic methods to introduce T-DNA based plasmids into plants: the *Agrobacterium tumefaciens* and the *Agrobacterium rhizogenes* mediated transfer. Using *A. tumefaciens* strains, transformed calli are obtained first, from which somatic embryos have to be induced which have to be regenerated to raise transgenic plants. This procedure can be carried out only by using so-called embryogenic plants. Using *A. rhizogenes* strains for infection, hairy roots grow on the infected part of the plant, and as a result a chimeric organism is formed in which only the hairy roots are transformed.

Since preliminary results showed that the plant (MN-1008) carrying the nn_1 mutation is not embryogenic under standard conditions, i.e. plants cannot be regenerated from the calli transformed by the *A. tumefaciens* mediated method, two strategies were chosen for the genetic complementation.

(i) Using the *Agrobacterium rhizogenes* transformation method, when the ability for the symbiotic nodule formation of the transformed hairy roots can be investigated. Transformed roots of most legume species are able to form nodules(55, 56), that is why, in the case of complementation, the formation of nodules might be expected on the roots of the mutant plants.

(ii) The non-embryogenic MN-1008 plant was crossed as described in the Example 1. with an individual of an embryogenic line (Regen SY) which was provided by Dr. Deborah A. Samac, University of Minnesota, MN, USA (57). After the selection and self-pollination of the embryogenic F1 individuals, the symbiotic phenotype of the individuals of the F2 population was determined as described in the Example 1., and two plants from the Nod$^-$ progeny (F2RN28/4, F2RN28/5) as well as two embryogenic F1 plants (F1RN28 és F1RN41) was used for the *A. tumefaciens* mediated transformation experiments.

Example 12A.

The cloning of the NORK gene into plant transformation vectors

The fragment carrying the NORK gene was cloned into several plant transformation vectors which have different copy number and provide different DNA-environment in the T-DNA that might affect the expression of the transformed gene.

The 8.5 kb NheI fragment carrying the NORK gene was cloned into the different plant transformation vectors from plasmid pBRC1660 which was constructed as follows: From the G20P5 clone (see Table 5.) constructed as described in Example 6., the insert was cut by the restriction endonucleases *Pae*I and *Sac*I and was ligated into the pOK12 (49) vector which was digested with the same enzymes and dephosphorilated by the CIP enzyme. From the obtained clone designated as pAT688 the insert was cleaved by the restriction endonucleases *Cla*I és *Sac*I and it was ligated into the vector pBlueScript II SK (Stratagene, 11011 North Torrey Pines Road, La Jolla, CA, USA). From the clone pBRC1660 obtained as described above (maintained in *E. coli* DH5α strain under the code BRC1660) the NORK gene can be deliberated by several enzymes. The pOK12 vector was provided by Dr. J. Messing (Waksman Institute, Piscataway, NJ, USA).

The 11.8 kb *ClaI* fragment harbouring the NORK gene with a 2.2 kb longer 5' (promoter) sequence was cloned into the different plant transformation vectors from plasmid pBRC1690 which was constructed as follows: The BAC clone 67A11 shown in Figure 5. was digested by ClaI enzyme, then the fragment was re-isolated from agarose gel as described in the Example 3. and was ligated into pBlueScript II SK vector which was digested by the same restriction endonuclease and dephosphorilated by the CIP enzyme.

The pPK459 vector which was used for the construction of the pBRC1678 clone was developed as follows: The *Hin*dIII-*Eco*RI fragment from the polylinker of the pSL301 plasmid (59) was cloned between the *Hin*dIII és *Eco*RI sites of the pGA471 vector described by An et al. (58). The pGA471 vector (58) and the pSL301 vector (59) were kindly provided by G. An (Washington State University, Pullman, WA, USA) and J. Brosius (Mt. Sinai School of Medicine, New York, NY, USA), respectively.

The pPR97 vector (60) used for the construction of clones pBRC1666 and pBRC1701 was provided by László Szabados (MTA, BRC, Szeged).

The pAT680 vector used for the construction of the pBRC1667 clone was constructed as follows: the T-DNA part of the pGREEN II vector described by Hellens et al. (61) was digested by HpaI enzyme and treated by the CIP enzyme and was ligated to an EcoRV fragment carrying the nopaline synthase gene promoter (nos) driven neomycin phosphotransferase II gene providing kanamycin resistance (nos-KmR casette), and the obtained clone was deposited in the strain collection under the name pAT678. After removing by EcoRV digestion from the carrier plasmid, the intron-containing *uidA* gene (62) coding for the β-glucuronodase gene and driven by 35S promoter of the cauliflower mosaic virus (35S-GUS-INT casette; 61) was built into the StuI digested CIP treated pAT678 vector. The T-DNA containing BglII fragment was cloned from the obtained pAT679 plasmid into the BamHI site of the broad host range vector pAT672 which was constructed from the replication origo of plasmid pBBR1MCS-4 (63) and the

kanamycin resistance gene of plasmid pK19 (64) as follows: The kanamycin resistance gene from plasmid pK19 was amplified as described in Example 4. using primers KMU (5'-GGCGATCGATAGACTGGGCGG) and KMD (5'-TCGTGATGGCAGGTTGGGCG), then the amplified fragment was cleaved by ClaI and reisolated from agarose gel as described in Example 3. The isolated fragment was cloned into the EheI and ClaI digested pBBR1MCS-4 vector. The original restriction enzyme cutting sites from the resulted pAT671 vector were removed in two steps as follows: The DNA was digested by *Bam*HI and *Bsp*119I, then *Bam*HI and *Cla*I enzymes and the digested DNAs were treated in the presence of 0,05 mM dNTP with the Klenow fragment of DNA polymerase I as described in Example 4. and ligated. The overhanging ends of the DNA resulted after the *Bam*HI and *Bsp*119I, as well as the *Bam*HI and *Cla*I digestions were filled in by the Klenow fragment which restored the recognition site (GGATCC) of *Bam*HI. Plasmids pK19 (64) and pBBR1MCS-4 (63) were kindly provided by D. Pridmore (Ciba-Geigy AG, Basel, Switzerland) and E. Kovach (Louisiana State University, Shreveport, LA, USA), respectively. Vector pGREEN II and casettes nos-KmR and 35S-GUS-INT can be obtained from the John Innes Centre, Norwich, England (detailed information: www.pgreen.ac.uk).

These cloning vectors (pPK459, pAT680, pPR97) were digested by restriction enzymes shown in the third column of Table 7., purified and dephosphorilated by the CIP enzyme as described in Example 4. After a second purification step 1 μg of vector DNA was ligated with 1 μg of the insert carrying the NORK gene. The insert carrying the NORK gene was deliberated from the plasmid pBRC1660 by enzymes shown in the third column of Table 7. then isolated from gel as described in Example 3. The ligated DNAs were transformed into *E. coli* strain DH5α, then after the checking of the transformant colonies (see Example 6A) the *E. coli* strains carrying the clones were deposited under the codes BRC1666, BRC1667, BRC1678 and BRC1701 (Table 7.).

The plasmids carrying the NORK gene (pBRC1666, pBRC1667, pBRC1678, pBRC1701) were purified from the *E. coli* strains and were transformed into the different *Agrobacterium rhizogenes* és *Agrobacterium tumefaciens* strains shown in Table 8. The transformations were carried out by electroporation as follows. Bacteria grown on solid LB medium containing 100 μg/ml of rifampicin antibiotics were inoculated into 5 ml of YENB (65) medium containing 100 μg/ml of rifampicin and were incubated overnight at 30 °C with good aeration. 200 ml of YENB medium was inoculated with this starter culture. The bacterium culture was grown with intensive aeration to late logarithmic phase (optical density at 600 nm, OD_{600}=0.6-0.8), then the cells were collected by centrifugation. The bacteria were washed at 0°C two times with 100-100 ml of sterile distilled water and two times with 10 ml of 1mM HEPES (pH=7) containing 10% of glicerol. Bacterial cells were resuspended at 0°C in 2-4 ml of sterile 1mM HEPES (pH=7) containing 10% of glicerol, and these electrocompetent cells were frozen in liquid nitrogen as 100 μl aliquots and stored at –80 °C till usage. 0.1-1 μg of plasmid DNA was added to the electrocompetent cells on ice, then the DNA was introduced into the cells at 1.25 kV/cm field strength with the help of to E.coli Pulser instrument (BioRad) according to the supplier's instructions. Immediatly after the electroporation, 1 ml of LB medium was added to the cells, then after an incubation at 30 °C for 120 minutes the cells were plated onto LB medium containing 100 μg/ml of rifampicin and antibiotics shown in Table 7.

Example 12B.

Transformation using *Agrobacterium rhizogenes* strains

The *A. rhizogenes* strains (BRC1673, BRC1675, BRC1679; Table 7.) carrying the NORK gene on plasmid can be introduced into the tissues of the Nod$^-$

mutant *M. sativa* plants by two ways: 1.) through the sectioned surface of seedlings' radicle and 2.) through the sectioned surface of shoots of mature plants.

Transformation via cutting the radicles of seedlings: The seeds of the MN-1008 and the Nod⁻ plants (NAB701, NAB809, NAB897, NAB968, NAB2792) carrying nn_1 mutation - which were identified and maintained as described in Example 1. - were sterilized and germinated on 1% water agar as desribed in Example 1. The rootlets of the 3-4 day old seedling were cut 5-10 mm below cotyledon and the wounded roots were dipped into a lawn of *A. rhizogenes* grown on solid LB medium. The *A. rhizogenes* strains (BRC1673, BRC1675, BRC1679) were streaked from the strain collection stored at –80 °C onto LB medium containing 100 μg/ml of rifampicin and antibiotics shown in Table 7. and were incubated at 30 °C for 3 days. The plants treated with the *Agrobacterium* strains were placed into squared (10x10 cm) Petri-dishes containing TM-1 medium (66) and were grown at 24°C with 16 hour light period and 10000 Lux light intenstity. After 5 days roots were appearing at the cut surface, and part of them were hairy root, i.e. transformed root.

2. Transformation through the sectioned shoots of mature plants: The young shoots of the MN-1008 and the Nod⁻ plants (NAB701, NAB809, NAB897, NAB968, NAB2792) identified and maintained as described in Example 1. that carry the nn_1 mutation in homozygous configuration were cut from the plants and placed immediately into tap water. The sectioned surfaces of the shoots were dipped into melted parrafine to avoid the entering of the chemicals used for sterilization into the vascular tissues. Then the shoots were sterilized as follows: they were washed in 70% ethanol for 5 seconds then immediately in 20% bleech containing 0.05 % Tween 20 for 90 seconds. After 3 washing in sterile destilled water the shoots were cut into pieces of 3-5 cm. After dipping the root-proximal surface into *Agrobacterium rhizogenes* lawn grown as described in the previous paragraph the distal end of the plants were stabbed into TM-1 medium and were incubated at 22°C with 16 hour light period and 10000 Lux light intenstity. From the 20. day

roots were appearing at the place of the wounding and some of them were transformed

Transformed roots could be identified by the so-called GUS-staining (67) because the T-DNA carries the uidA gene coding for the *β*-glucuronidase enzyme. The roots were stained fro the GUS activity as follows: root pieces were incubated at 37°C in dark for 12-24 hours in a buffer containing 100 mM TRIS (pH=7.4), 50 mM NaCl, 2 mM spermidine, 2 mM $K_3Fe(CN)_6$, and 2 mM X-GlcU (5-bromo-4-chloro-3-indol β-D-glucuronic acid) dye. The *β*-glucuronidase enzyme expressed in the transformed roots cleaves the dye and as a result a blue product precipitated in the cells is formed which can be detected both by eyes and by microscope. The *A. rhizogenes* strains used for transformation and the transformation efficiency is shown in Table 9. After the transformation 4 transformed roots were obtained, however, no nodule formed in the presence of compatible *S. meliloti*. The reason for this results might be that the T-DNA of the Ri plasmid harbours genes coding for enzymes involved in plant hormone biosynthesis and the expression and biological effect of these genes in the plant cells inhibit nodule formation (Á. Kondorosi, personal communication).

Example 12C.

Transformation using *Agrobacterium tumefaciens* strains

The *A. tumefaciens* transformation was carried out as follows: Twenty pieces of healthy, dark-green leaves were cut from the alfalfa plant to be transformed and placed immediately into Petri dishes containing tap-water. The leaves were sterilized as follows: after washing with 70 % ethanol for 5 seconds, they were placed immediately into a 20 % of bleech containing 0.05 % of Tween 20, and washed for 90 seconds. This was followed by three washing steps in sterile destilled water. The edges and the central vascular tissues of the sterilized

leaves were removed and the two pieces were split in two. These leaf pieces - with all the edges cut - were placed into SHO medium containing *A. tumefaciens* which was prepared as follows: The *A. tumefaciens* strains (BRC1677, BRC1680, BRC1681, BRC1707; Table 8.) stored at –80 °C were streaked on solid medium containing 100 μg/ml of rifampicin, as well as the proper antibiotics shown in Figure 7. and incubated at 30°C. A single colony was inoculated by loop into 3 ml of liquid YEP medium (in 20 ml tube) and the bacteria were grown by rolling (to provide aeration) to the stacioner phase (24 óra). The bacteria were collected by centrifugation as described earlier, the supernatant was poured away, and the bacteria were suspended in 12 ml of SHO medium. After keeping in the *Agrobacterium* suspension for 20 minutes the leaves were removed and the excess of suspension was blotted by sterile blotting paper, then the leaf pieces were placed onto B5h in Petri dishes (maximum 16 leaf pieces per plate). The plates were sealed by air-permeable bands and placed to 24°C and 16 hour light period with 10000 Lux light intenstity. After 1 week the leaves were removed from the medium, washed 3 times with sterile distilled water and placed onto B5hKmCb medium then were incubated as described above. After 3 weeks the leaf pieces forming calli were placed onto B5hKmCb medium and were further incubated as described above. The appearing dark green embryos were transferred onto MMSCb medium and were further incubated as described above. The plantlets forming roots and shoots were transferred into jars containing MMSCb medium where the putative transformants were raised. The developed plants were propagated in vitro as follows: a piece of the shoot containing 3-4 pair of leaves was cut under sterile conditions, placed into MMSCb medium in a jar and incubated as described above.

After strengthening of the shoots and roots of the propagated plants, DNA was isolated from the leaves of the plants as described in Example 2., then to detect the transformation events PCR reactions were carried out as described in the Example 3. by using primers specific for the *nptII* gene providing resistance

against kanamycin (NPT-U: 5'-ACCCAGCCGGCCACAGTCG-3', NPT-D: 5'-GGGCGCCCGGTTCTTTTTG-3') and for the uidA gene encoding the β-glucuronidase enzyme (GUS-U: 5'-TTATGCGGGCAACGTCTGGTAT-3', GUS-D: 5'-AGTCCCGCTAGTGCCTTGTCC-3') that are within the T-DNA.

After transfering to nitrogen-free Gibson medium, the plants were infected with *S. meliloti* strain 41 and the process of nodulation was followed. Transformed derivatives of RN28 and RN41 were self-pollinated then germinated as described in Example 1. and we checked their nodule formation ability.

The results of the transformation experiments by using the *A. tumefaciens* strains are summarized in Table 10.

The composition of the YEP medium:

10 g/l Protease Peptone (Difco Laboratories)

10 g/l Bacto yeast extract (Difco Laboratories)

5 g/l NaCl

Sterilization: 121 C^o, 20 minutes.

The composition of the LB medium:

10 g/l Bacto-tryptone (Difco Laboratories)

5 g/l Bacto yeast extract (Difco Laboratories)

5 g/l NaCl

pH=7.5

Sterilization: 121 C^o, 20 minutes.

The composition of the YENB medium:

8 g/l Bact Nutrient Broth (Difco Laboratories)

7.5 g/l Bacto yeast extract (Difco Laboratories)

Sterilization: 121 C^o, 20 minutes.

The composition of the SHO medium:

Schenk and Hildebrant salt:

KNO_3	2500 mg/l
$MgSO_4 \cdot 7H_2O$	400 mg/l
$NH_4H_2PO_4$	300 mg/l
$CaCl_2 \cdot 2H_2O$	200 mg/l
$MnSO_4 \cdot H_2O$	10 mg/l
H_3BO_3	5 mg/l
$ZnSO_4 \cdot 7H_2O$	1 mg/l
KI	1 mg/l
$CuSO_4 \cdot 5H_2O$	0.2 mg/l

$NaMoO_4 \cdot 2H_2O$	0.1 mg/l
$CoCl_2 \cdot 6H_2O$	0.1 mg/l
$FeSO_4 \cdot 7H_2O$	15 mg/l
Na_2EDTA	20 mg/l

Schenk and Hildebrant vitamine solution:

myo-inositole	1000 mg/l
Nicotinic acid	5 mg/l
Thiamine·HCl	5 mg/l
Pyridoxine·HCl	0.5 mg/l

30 g/l saccharose

0.5 g/l MES (Sigma)

pH= 7.5 (adjusted with KOH)

Sterilization: 121 C^o, 20 minutes.

The composition of the B5h medium:

1 liter of B5 soulution

30 ml of B5 amino acid stock solution

1 ml of B5 hormone stock solution

The composition of the B5 solution:

Gamborgs' B5 salts:

KNO_3	2500 mg/l
$MgSO_4 \cdot 7H_2O$	250 mg/l
$(NH_4)_2SO_4$	134 mg/l
$CaCl_2 \cdot 2H_2O$	150 mg/l
$NaH_2PO_4 \cdot H_2O$	150 mg/l
$MnSO_4 \cdot H_2O$	13.5 mg/l
H_3BO_3	3 mg/l

$ZnSO_4 \cdot 7H_2O$	2 mg/l
KI	0.75 mg/l
$CuSO_4 \cdot 5H_2O$	0.025 mg/l
$NaMoO_4 \cdot 2H_2O$	0.25 mg/l
$CoCl_2 \cdot 6H_2O$	0.025 mg/l
$FeSO_4 \cdot 7H_2O$	27.8 mg/l
Na_2EDTA	37.3 mg/l

Gamborgs' B5 vitamine solution:

myo-inositol	100 mg/l
Nicotinic acid	1 mg/l
Thiamine·HCl	10 mg/l
Pyridoxine·HCl	1 mg/l

0.5 g/l proline

40 g/l saccharose

pH= 5.7 (adjusted with KOH)

8 g/l Phytoagar (GIBCO BRL)

Sterilization: 121 C^o, 20 minutes.

Before plating sterile amino acid and hormone stock solutions are added.

B5h amino acid stock solution:

6.65 g/250 ml L-glutamine

0.83 g/250 ml serine

0.004 g/ 250 ml adenine

0.083 g/250 ml glutathione

Filter sterilization. 30 ml of amino acid stock solution is added to 1 l of B5h medium before plating

B5h hormone stock solution:

1 mg/ml 2, 4-D-(2,4-dichlore-phenoxy-acetic acid, GIBCO BRL)

0.1 mg/ml kinetin (GIBCO BRL)

Filter sterilization. 1 ml of homone stock solution is added to 1 l of B5h medium before plating

The composition of the B5hKmCb medium:

B5h medium containing 25 mg/l of kanamycine and 500 mg/l of carbenicilline.

The composition of the MMSCb medium:

4.3 g/l Murashige and Skoog salt (GIBCO BRL)

1 ml/l 1000x Nitsch and Nitsch vitamine stock solution:

myo-inositol	100 mg/ml
glycine	2 mg/ml
Nicotinic acid	5 mg/ml
Pyridoxine·HCl	0.5 mg/l
Thiamine·HCl	0.5 mg/l
Folic acid	5 mg/l
Biotine	0.05 mg/l

30 g/l saccharose

pH= 5.7 (adjusted with KOH)

8 g/l Phytoagar (GIBCO BRL)

Sterilization: 121 C°, 20 minutes.

500 mg/l of carbenicilline is added after sterilization and before plating.

Example 13.

The introduction of the NORK gene of *M. truncatula* into other (leguminous and non-leguminous) plants

The *Agrobacterium tumefaciens* strains described in Example 12. (BRC1677, BRC1680) carrying the NORK gene of *M. truncatula* were used for the introduction of the NORK gene into the legume plant vetch (*Vicia villosa*) and the non-legume tobacco (*Nicotiana tabacum* cv. Small Havanna SR1) plant. The transformations were performed as described in Example 12C with the modification that in the case of *Vicia* 2 mg/l of 2,4-D and 0.2 mg/l of kinetin was used, while the pieces of the tobacco leaves were placed onto MS medium (68) containing 1 mg/l of benzyl-aminopurine (BAP) and 0.1 mg/l of naphtyl-acetic acid (NAA), as well as antibiotics (100 mg/l of kanamycin and 500 mg/l of carbenicillin).

After the transformations six *N. tabacum* and eight *V. villosa* plants originated from independent transformation events were regenerated (Table 11.). Total DNA from the transformed plants was isolated and PCR amplification using the Pr-RKU4---Pr-RKD5 primerpair (Figure 9.) was carried out as described in Examples 2. and 9., respectively. The sequence of amplified products (exon1-exon2 of the NORK gene) were determined as described in Example 8. The sequencing results revealed that all sequences determined from the transformed plants were identical to the DNA sequence of the *M. truncatula* NORK gene, which means that the transgenic plants contains the NORK gene of *M. truncatula*. Proteins from the roots of transformed tobacco plants were isolated as described in Example 14. and the presence of the NORK protein in the transformants was demonstrated by dot blot hybridization. These results show that the NORK gene is expressed in *N. tabacum*. The results of the transformation experiments and the characterization of the transformants are shown in Figure 32.

The composition of the MS medium:

Murashige and Skoog salts:

KNO_3	1900 mg/l
$MgSO_4 \cdot 7H_2O$	70 mg/l
NH_4NO_3	650 mg/l
$CaCl_2 \cdot 2H_2O$	40 mg/l
$KH_2PO_4 \cdot H_2O$	70 mg/l
$MnSO_4 \cdot H_2O$	22.3 mg/l
H_3BO_3	8.2 mg/l
$ZnSO_4 \cdot 7H_2O$	8.6 mg/l
KI	0.83 mg/l
$CuSO_4 \cdot 5H_2O$	0.025 mg/l
$NaMoO_4 \cdot 2H_2O$	0.25 mg/l
$CoCl_2 \cdot 6H_2O$	0.025 mg/l
$FeSO_4 \cdot 7H_2O$	27.8 mg/l
Na_2EDTA	37.3 mg/l

Murashige and Skoog vitamine solution:

myo-inositol	100 mg/l
Nicotinic acid	0.5 mg/l
Thiamine·HCl	0.1 mg/l
Pyridoxine·HCl	0.1 mg/l
Glycine	2 g/l
Saccharose	20 g/l

pH= 5.7 (adjusted with KOH)

Phytoagar (GIBCO BRL) 8 g/l

Sterilization: 121 C^o, 20 minutes.

Example 14

Immunological detection of the NORK protein

In order to prove the presence of NORK protein in the cells of alfalfa, and in the transformed tobacco, a non-legume plant, two exons (2nd and 3rd exons) of the N-terminal (5') part of the NORK gene were expressed in *Escherichia coli*. Antibodies were produced against the purified proteins and the NORK protein was detected by immunological method (dot blot analysis).

Example 14A.

Cloning of the DNA fragment coding the N-terminal part of *M. truncatula* A17 NORK protein into the expression vector pTrcHisA

The pTrcHis (A, B and C) (Invitrogen BV Groningen, The Netherlands) expression vectors are made possible to express recombinant protein containing six histidine (His-tag) and a recognition site of enterokinase enzyme in fusion. The His-tag allows us to purify the recombinant protein in a single-step purification procedure (recommended by the suppliers). For this reasons this system was used to express the extracellular part of the NORK protein.

A DNA fragment of the *M. truncatula* NORK cDNA sequence described in Example 9 and showed in Figure 10 was produced by PCR amplification using the RKEx2U and Pst-A3 primers (Figure 9.) as it is described in Example 9A. The 957 bp long amplified cDNA fragment coded 319 amino acids (amino acids from 33 to 352) of the supposed extracellular part of the NORK protein. pTrcHisA vector was used for cloning to fuse the sequence of the NORK protein portion to be expressed and the His-tag in frame. The pTrcHisA vector and the amplified cDNA fragment were digested by *BamHI* and *PstI* restriction enzymes (Fermentas AB) according the supplier's instructions. The fragment and the vector prepared in this way were ligated (construct pTrcHisA::dNORK) as it is described in Example 4. The ligation is transformed into *E. coli* TOP10 competent cells detailed also in

Example 4. After transformation and plating, plasmid DNA was isolated from the ampicillin resistant colonies using the QIAGEN Plasmid Isolation Kit (see Example 4.). The frame and the fidelity of the sequence of the insert were checked by sequence analysis (see Example 8.). The amplified and correctly inserted clone was termed TOP10(pTrcHisA::dNORK).

Example 14B

Expression of the extracellular part of the NORK protein in *E. coli*

The recombinant protein (rNORK) produced by the Xpress System Protein Expression system (construct pTrcHisA::dNORK) was purified by the Xpress System Protein Purification (Invitrogen BV Groningen, The Netherlands) according to the manufacturer's instructions. Overnight culture of the clone TOP10(pTrcHisA::dNORK) (see Example 14A.) grown in 2 ml of LB medium containing ampicillin in 50 μg/ml concentrations was diluted 250 times in 50 ml of the same medium. The culture was grown at 37°C with vigorous shaking to an OD_{600}=0.6. Then the expression of protein was induced by adding 0.5 ml IPTG solution (100 mM) and the culture was shaked for 5 more hours. Bacterial suspension was collected by centrifugation (centrifuge: Sorvall RC5, GSA rotor with the proper tubes, 4°C, 10 min, 8k rpm) than the bacterial pellet was resuspended in 10 ml of buffer (recommended by the kit: 20 mM NaH_2PO_4, 500 mM NaCl; pH 7.8). Disruption of bacteria was made by sonication 3 times for 10 sec using the smallest head of MSE Ultrasonic Disintegrator. The supernatant of the disrupted and collected bacterial suspension was bound to affinity column through the six histidine amino acid residue present in the fusion protein. Bacterial proteins were removed by three washing steps using solutions with decreasing pH (20 mM NaH_2PO_4, 500 mM NaCl; pH 7.8; 6.0 and 5.5). The recombinant protein was eluted from the column by 5 ml elution buffer of the kit (20 mM NaH_2PO_4, 500 mM NaCl; pH 4.0) having 1 mg/ml protein concentration. The eluted fusion protein was used directly to produce antibodies in rabbits.

Example 14C

Producing polyclonal antibodies against the extracellular part of the NORK protein

Three, 4 months-old rabbits were injected by native protein in 1 ml final volume dissolved in PBS (69) and purified as described in Example 14B, respectively. Approximately 100 μg of total purified protein (antigen) was injected subcutan into four different sites of the neck. For the primary injection 50% complete Freunds adjuvant (CFA) (cat. no: F-5881 Sigma-Aldrich Kft. Budapest) and 50% recombinant protein dissolved in PBS were used. Before the primary injection the rabbits were bled and the gained sera were used later as a negative (isotype) control.

After the fourth and seventh weeks of the primary injection boost injection was performed to achieve a heigher state of immunity (hyperimmunization). For the second injection 10% CFA + 40% incomplete Freunds adjuvant (IFA) (cat. no: F-5506 Sigma-Aldrich Kft. Budapest) and 50% recombinant protein dissolved in PBS were used. Ten days later the antibody production was checked. Serum was prepared from 1-5 ml blood obtained from ear's vein. The obtained blood was kept at room temperature for 4 hours and after coagulation it was centrifuged (2700g, 4°C, 10 min). A part of the supernatant (serum) was kept at 4°C untill utilization and the rest was stored at -20°C in 100 μl aliquots. The antibody content of the antisera was checked by ELISA (Enzyme-Linked Immunosorbent Assay) and dot blot analysis. The rabbits were kept alive and were injected again on the 7th week after the primary injection. For the third injection 50% IFA and 50% recombinant protein dissolved in PBS were used. On the 10th day after the third injection, the rabbits were bled and the samples were checked again by ELISA and dot blot analysis. Then 80 ml blood was taken from the rabbits.

Example 14D

ELISA

500 μg recombinant protein dissolved in PBS per well was plated into 96-well microtiter plate and incubated at 37°C for 2 hours. Then the plate was washed three times by washing buffer (0.05% Tween-20, 0.5 M NaCl in PBS) and was blocked by 0.5% gelatine (1 hr, 37°C). After three washes the plate was incubated at 37°C for 1 hour in the presence of 100 and 1000-fold diluted rabbit anti sera in 50μl washing buffer. After three repeated washes the plate was incubated (1 hr, 37°C) with the second antibodies diluted in 50μl washing buffer for 10000-fold. Horseradish peroxidase conjugated anti-rabbit IgG antibody (cat. No: A-0545 Sigma-Aldrich Kft. Budapest) was used as a secondary antibody. After four washes the plate was developed by 0.5 mg/ml OPD substrate (cat. no: P-5412 Sigma-Aldrich Kft. Budapest). OPD was dissolved in PBS containing 0.1 M citrate than 5 μl 30% H_2O_2 was added to it. After blocking the reaction by 4 M sulphuric acid (50 μl/well), the plates were read with a microplate ELISA Reader (Labsystem Multiscan Biochromatic) at 492 nm. The most specific antibody selected based on the results of ELISA test was used in 1000-fold dilution to investigate the extracts purified from roots of different plants.

Example 14E.

Protein purification from the roots of mutant (Nod^-) and wild type (Nod^+) *Medicago sativa* and transformed and control *Nicotiana tabacum* plants

Purification of proteins isolated from the roots of MN-1008 (Nod^-) and NAB814 (Nod^+) *Medicago sativa* as well as control (non-transformed) and transformed *Nicotiana tabacum* plants (see Example 13) were performed by the slightly modified methods of Huang and Berry (70) as well as Cheong and Hahn (59). 1 g fresh roots were homogenized in 1 ml homogenisation buffer (25 mM Tris-HCl pH 7.0, 30 mM $MgCl_2$, 2 mM dithiotreitol (DTT), 0.2 mM

phenylmethylsulfonyl fluoride (PMSF)) in the presence of quartz sand on ice. The homogenate was filtered through four layers of cheesecloth (commercially available) and centrifuged at 6000g (4°C, 15 min). Supernatant and the crude membrane fraction obtained by centrifugation of the supernatant (20000g, 4°C, 30 min) were used for dot blot analysis.

Example 14F

Dot blot analysis of proteins purified from the roots of *Medicago sativa* and *Nicotiana tabacum* using specific antibodies against the NORK protein

Ten μg of protein samples purified from alfalfa and tobacco plants (the filtered and centrifuged supernatant of plant homogenized samples described in Example 14E) were dot-blotted onto nitrocellulose filter (Hybond-ECL cat. no: RPN303D, Amersham Pharmacia Biotech Inc, Little Chalfont, Buckinghamshire, England) (Figure. 32.). Dot blot analysis was performed with the 1000x diluted rabbit antisera as detailed below. Filters were blocked by blocking solution (2.5% milk powder, 1% polyvinyl-pyrrolidone (PVP), 0.1% Tween-20 in PBS) at room temperature for 1 hour. The filters were incubated in the same solution containing the generated antibodies at room temperature for another 1 hour. The filters were washed three times each for ten minutes in 50 ml washing solution (0.1% Tween-20 in PBS). The filters were incubated further with the horseradish peroxidase-conjugated anti-rabbit IgG (5000x diluted in washing solution) at room temperature for 1 hour. After three washing steps the filters were developed by chemiluminescent reaction. After incubation of the filters in developing solution (100 mM Tris pH 8.5, 450 μM coumaric acid, 2.3 mM luminol and 0.03% H_2O_2) for 1 min, they were visualized by an X-ray film exposure (Sterling Diagnostic Imaging Inc., Newark, DE 19714 USA) (Figure 32.). The positive signal detected in the case of the Nod^+ and Nod^- alfalfa as well as of the transformed tobacco plant containing the NORK gene demonstrated the reaction of the NORK protein with the produced specific antibody.

Literature references:

1. Kiss, G.B. 1986. A szimbiotikus nitrogénkötés fokozásának lehetoségei. Növénytermelés 35:351-361.

2. Baldani, J. I., Caruso, L., Baldani, V. L. D., Goi, S. R., Dobereiner, J. 1997. Recent advances in BNF with non-legume plants. Soil Biology & Biochemistry 29: 911-922.

3. Pueppke, S. G. and Broughton, W. J. 1999. Rhizobium sp. Strain NGR234 and R. fredii USD257 share exceptionally broad, nested host range. Mol. Plant Microb. Interact. 12:293-318.

4. Norris, D.O. 1956. Legumes and the Rhizobium symbiosis. Emp. J. Exp. Agric. 24:247-270.

5. Schultze, M., Kondorosi, E., Ratet, P., Buiré, M., Kondorosi, A. 1994. Cell and molecular biology of Rhizobium-plant interaction. Int. Rev. Cytology 156:1-75.

6. Horváth, B., Kondorosi, E., John, M. , Schmidt, J., Török, I., Györgypál. Z., Barabás, I., Wieneke, U., Schell., J., Konsorosi, A. 1986. Organization, structure and symbiotic function of Rhizobium meliloti nodulation genes determining host specificity for alfalfa. Cell 46:335-343.

7. Schultze, M. , Kondorosi, A. 1995. What makes nodulation signals host-specific? Trends in Microbiology 3:370-372.

8. Long, S. R. 1989. Rhizobium-legume nodulation: life together in the underground. Cell 56: 203-214.

9. Györgypál, Z., Iyer, N., Kondorosi, A. 1988. Three regulatory nodD alleles of divergent flavonoid-specificity are involved in host-dependent nodulation by Rhizobium meliloti. Mol. Gen. Genet. 212:85-92.

10. Bergman, K., Gulash-Hofee, M. , Hoverstadt, R. E., Larosiliere, R. C., Ronco, P. G., Su, L. 1988. Physiology os behavior mutants of Rhizobium meliloti: evidence for a dual chemotaxis pathway. J. Bacteriol. 170: 3249-3254.

11. Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Prome, J. C. and Denarie, J. 1990. Symbiotic host-specificity of Rhizobium-

meliloti is determined by a sulfated and acylated glucosamine oligosaccharide signal. Nature 344: 781-784.

12. Horváth, B., Bachem, C.W.B., Schell, J., Kondorosi, A. 1987. Host specific regulation of nodulation genes in Rhizobium memliloti is mediated be a plant signal, interacting with the nodD gene product. EMBO J. 6:841-848.

13. Kondorosi, E., Gyuris, J., Schmidt, J., John, M. , Duda, E., Hoffmann, B., Schell, J., Kondorosi, A. 1989. Positive and negative control of nod gene expression in Rhizobium meliloti is required for optimal nodulation. EMBO J. 8:1331-1340.

14. Caetano Annoles, G. and Gresshoff, P.M. 1991. Plant genetic control of nodulation. Ann. Rev. Microbiol. 45:345-382.

15. Bhuvanesvari, T.V., Solheim, B. 1985. Root hair deformations in the white clover/Rhizobium trifolii symbiosis. Physiol. Plant. 63:25-34.

16. Peters, N.K., Frost, J.W., Long, S. 1986. A plant flavon, luteolin, induces expression of Rhizobium meliloti nodulation genes. Science 233:977-980.

17. Rolfe, B. G. and és Gressoff, P.M. 1988. Genetic analysis of legume nodulation. Ann. Rev. Plant Physiol. Plant Mol. Biol. 39:297-319.

18. Niebel, A., Gressent, F., Bono, J. J., Ranjeva, R., Cullimore, J. 1999. Recent advances in the study of Nod factor perception and signal transduction. Biochimie 81: 669-674.

19. Truchet, G., Barker, D. G., Camut, S., de Billy, F., Vasse, J., Huguet, T. 1989. Alfalfa nodulation in the absence of Rhizobium. Mol. Gen. Genet. 219:65-68.

20. Freiberg, C., Fellay, R., Bairoch, A., Broughton, W. J., Rosenthal, A., Perret, X., 1997. Molecular basis of symbiosis between Rhizobium and legumes. Nature 87: 394-401.

21. Covitz, P. A., L. S. Smith, Long, S. R. 1998. Expressed sequence tags from a root-hair-enriched Medicago truncatula cDNA library. Plant Physiology 117: 1325-1332.

22. Gyorgyey, J., Vaubert, D., Jimenez-Zurdo, J. I., Charon, C., Troussard, L., Kondorosi, A., Kondorosi, E. 2000. Analysis of Medicago truncatula nodule expressed sequence tags. Molecular Plant-Microbe Interactions 13: 62-71.

23. Downie, J. A. and Walker, S. A. 1999. Plant responses to nodulation factors. Current Opinion in Plant Biology 2: 483-489.

24. Csanádi, G., Szécsi, J., Kaló, P., Kiss, P., Endre, G., Kondorosi, A., Kondorosi, E., Kiss, G.B. 1994. ENOD12, an early nodulin gene, is not required for nodule formation and efficient nitrogen fixation. Plant Cell 6: 201-213.

25. Schauser, L., A. Roussis, Stiller, J., Stougaard, J. 1999. A plant regulator controlling development of symbiotic root nodules. Nature 402:191-195.

26. Tanksley, S.D., Ganal, M. W., and Martin, G.B. 1995. Chromosome landing: a paradigm for map-based gene cloning in plants with large genomes. Trends in Genetics: 11: 63-68.

27. Peterson, M. A. and Barnes, D.K. 1981. Inheritance of ineffective nodulation and non-nodulation traits in alfalfa. Crop Science 21:611-616.

28. Barnes, D. K., Vance, C.P., Heichel, G.H., Peterson, M. A., Ellis, W.R. 1988. Registration of a non-nodulation and three ineffective nodulation alfalfa germplasms. Crop Sci. 28:721-722

29. Catoria, R., Galera, C., de Billy, F., Penmetsa, V., Journet, E-P., Maillet, F., Rosenberg, C., Cook, D., Gough, C., Denarie, J. 2000. Four genes of Medicago tuncatula controlling components of a Nod factor transduction pathway. The Plant Cell 12:1647-1665.

30. Walker, S.A., Viprey, V., and J. Downie, A.J. 2000. Dissection of nodulation signaling using pea mutants defective for calcium spiking induced by Nod factors and chitinoligomers. PNAS 97: 13413-13418.

31. Parniske, M. , Coomber, S., Kistner, C., Mulder, L., Pitzschke, A., Stougaard, J., Szczyglowski, K., Webb, J., Stracke, S. Plant Gnetics of

symbiosis. In Abstract book of the EU conference of Molecular Genetics of Model Legumes, JIC, Norwich, June 24-28, 2000.

32. Dudley, M. E., and Long, S.R. 1989.A non-nodulating alfalfa mutant display neither root hair curling nor early cell division in response to Rhizobium meliloti. Plant Cell 1:65-72.

33. Ehrhardt, D. W., Wais, R., Long, S. R. 1996. Calcium spiking in plant root hairs responding to Rhizobium nodulation signals. Cell 85: 673-681.

34. Ambudkar, S. V., Sauna, Z. E., Muller, M. M., Kerr, K. M., Smith, M. M. 2000. Biochemical mechanism of action of multidrug resistance-linked ABC transporters. Chemistry and Physics of Lipids 107:1-15.

35. Schwiebert, E. M., Benos, D. J., Fuller, C. M. 1998. Cystic fibrosis: A multiple exocrinopathy caused by dysfunctions in a multifunctional transport protein. American Journal of Medicine 104: 576-590.

36. Song, W. Y., Wang, G. L., Chen, L. L., Kim, H. S., Pi, L. Y., Holsten, T., Gardner, J., Wang, B., Zhai, W. X., Zhu, L. H., Fauquet, C., Ronald, P. 1995. A Receptor Kinase-Like Protein Encoded by the Rice Disease Resistance Gene, Xa21. Science 270: 1804-1806.

37. Li, J. M. and Chory, J. 1997. A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. Cell 90 929-938.

38. Trotochaud, A. E., Hao, T., Wu, G., Yang, Z., Clark, S. E. 1999. The CLAVATA1 receptor-like kinase requires CLAVATA3 for its assembly into a signaling complex that includes KAPP and a rho-related protein. Plant Cell 11: 393-405.

39. Kiss, G.B., Kereszt, A., Kiss, P., Endre, G. 1998. Colormapping: a non-mathematical procedure for genetic mapping. Acta Biologica Hungarica 49:47-64.

40. Kaló, P., Endre, G., Zimányi, L., Csanádi, G., Kiss, G.B. 2000. Construction of an improved linkage map of diploid alfalfa (Medicago sativa). Theor. Appl. Genet. 100:641-657.

41. Gibson, A.H. 1980. Methods for legumes in glasshouses and controlled environment cabinets. In: Methods for evaluating biological Nitrogen Fixation. Bergersen, F.J. ed., John Wiley and Sons Ltd. p.139-184.

42. Szende, K, und Ördögh, F 1960. Die Lysogenie von Rhizobium meliloti. Naturwissenshaften 47:404-405.

43. De Lajudie, P., Willems, A., Pot, B., Dewettinck, D., Maestrojuan, G., Neyra, M. , Collins, M. D., Dreyfus, B., Kersters, K., and Gillis, M. 1994. Polyphasic taxonomy of rhizobia: Emendation of the genus Sinorhizobium and description of Sinorhizobium comb. nov., Sinorhizobium saheli sp. nov. and Sinorhizobium teranga sp. nov. Int. Journal of Systemic Bacteriol. 44:715-733.

44. Orosz L, Sváb Z, Kondorosi Á, Sík T 1973. Genetic studies on rhizobiophage 16-3. I. Genes and functions on the chromosome. Mol Gen Genet 125:341-350.

45. Michelmore, R.W., Paran, I., and Kesseli, R.V. 1991. Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. Proc. Natl. Acad. Sci. USA, 88:9828-9832.

46. Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acids Res. 18:6531-6535.

47. Kiss, G.B., Csanádi, G., Kálmán, K., Kaló, P., Ökrész, L. 1993. Construction of a basic genetic map of Medicago using RFLP, RAPD, Isozyme and morphological markers. Mol Gen Genet 238:129-137.

48. Church, G. M. and Gilbert, W. 1984. Genomic sequencing. PNAS 81: 1991-1995.

49. Maniatis, T., Fritsch,E.F., Sambrook, J. 1982. Molecular Cloning; A Laboratory Manual; Cold Spring Harbor Laboratory. 447.oldal.

50. Vieira J., Messing J. 1991. New pUC-derived cloning vectors with different selectable markers and DNA replication origins. Gene 100:189-194.

51. Turner SR, Ireland R, Morgan C, Rawsthorne S 1992. Identification and localization of multiple forms of serine hydroxymethyltransferase in pea (Pisum sativum) and characterization of a cDNA encoding a mitochondrial isoform. Journal of Biol Chem 267:13528-13534.

52. Woo, S. S., Jiang, J. M. , Gill, B. S., Paterson, A. H. and Wing, R. A. 1994. Construction and characterization of a bacterial artificial chromosome library of Sorghum bicolor. Nucleic Acids Research 22: 4922-4931.

53. Nam YW, Penmetsa RV, Endre G, Uribe P, Kim DJ, Cook DR 1999. Construction of a bacterial artificial chromosome library of Medicago truncatula and identification of clones containing ethylene response genes. Theor. Appl. Genet. 98:638-646.

54. Koncz, C., and Rédei, G. 2000. A molekuláris genetika eszközei: Agrobacterium T-DNS és Arabidopsis. Molekuláris növénybiológia C. Könyvben, szerkesztők: Balázs E., Dudits D. Akadémiai Kiadó. 323-452. oldal.

55. Quandt, H.-J., Pühler A.,and Broer I. 1993. Transgenic root nodules of Vicia hirsuta: A fast and efficient system for the study of gene expression in indeterminate-type nodules. Mol. Plant-Microbe Interact. 6:699-706.

56. Stougaard, J. 1995. Agrobacterium rhizogenes as a vector for transforming higher plants. Application in Lotus corniculatus transformation. Methods Mol. Biol. 49:49-61.

57. Bingham, E. T., Hurley, L. V., Kaatz, D. M., and Saunders, J. W. 1975. Breeding alfalfa which regenerates from callus tissue in culture. Crop Sci. 15:719-721.

58. Brosius, J. 1989. Superpolylinkers in cloning and expression vectors. DNA. 8:759-777.

59. An, G., Watson, B. D., Stachel, S., Gordon, M. P., Nester, E.W. 1985. New cloning vehicles for transformation of higher plants. EMBO J. 4:277-284.

60. Szabados, L., Charrier, B., Kondorosi, A., de Bruijn, F., Ratet, P. 1995. New plant promoter and enhancer testing vectors. Mol. Breeding 1:419-423.

61. Hellens, R.P., Edwards, E. A., Leyland, N. R., Bean, S. Mullineaux, P.M. 2000. pGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. Plant Mol. Biol. 42: 819-832.

62. Vancanneyt, G., Schmidt, R., O'Connor-Sanchez, A., Willmitzer, L., Rocha-Sosa, M. 1990. Construction of an intron containing marker gene: Splicing of the intron in transgenic plants and its use in monitoring early events in Agrobacterium-mediated transformation. Mol. Gen. Genet. 220:245-250.

63. Kovach, M. E., Phillips, R. W., Elzer, P. H., Roop, R. M., Peterson, K. M. 1994. pBBR1MCS: a broad-host-range cloning vector. Biotechniques.16:800-802.

64. Pridmore, R. D. 1987. New and versatile cloning vectors with kanamycin-resistance marker. Gene 56:309-312.

65. Sharma, R. C., ans Schimke, R. T. 1996. Preparation of electrocompetent E. coli using salt-free growth medium. Biotechniques. 20:42-4.

66. Shahin, E. A. 1985. Totipotency of tomato protoplasts. Theor. Appl. Genet. 69:235-240.

67. Jefferson, R. A. 1989. The GUS reporter gene system. Nature 342:837-838.

68. Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

69. Coico, R., Coligan, J.E., Kruisbeek, A.M., Margulies, D.H., Shevach, E.M., and Strober, W. Current Protocols in Immunology 1994. John Wiley & Sons Inc. USA.

70. Huang, L. S. and Berry, E. A. 1990. Purification and characterization of the proton translocating plasma membrane ATPase of red beet storage tissue. Biochim Biophys Acta 1039:241-52.

71. Cheong, J. J., and Hahn, M. G. 1991. A specific, high-affinity binding site for the hepta-beta-glucoside elicitor exists in soybean membranes. Plant Cell 3:137-47.

CLAIMS

1. Nucleotides sequence coding for the polypeptide having NOD region specific receptor kinase (NORK) gene activity.

2. Nucleotide sequence coding for the amino acid sequence of Figure 17.

3. Nucleotide sequence coding for the amino acid sequence of Figure 12.

4. Nucleotide sequence coding for the amino acid sequence of Figure 30.

5. Nucleotide sequence coding for the amino acid sequence of Figure 31.

6. The nucleotide sequence according to any of claims 1 to 5 which is a genomic or synthetic DNA molecule:

CCATATTTTAACAATATTCTTTCTTCTACAAGGGTATAACTTTTATACAAGTTCACTATA
TTATAGGATTGATCAAGGTTCATTTTTTCTTTCTTTGAAAAATCTCTAAGGGGTGTGGTT
TCCAAGGCAGAAAATGAAATAGAATGCAGAAGAATTTGTATGGTACTATAAAGGGAAGAT
GAAAAGTTAGTTAGCATGGATTCAAGTTTGATAACCCTTTGGGGTAAAATCTCTTTCAGA
TTATGATGGAGCTACAAGTTATTAGGATATTTAGATTGGTTGTGGCATGTGTTCTTTGTT
TGTGTATATTTATCAGATCAGCTTCTTCTGCAACTAAAGGGTTTGAGAGCATATCATGTT
GTGCTGATTCCAATTACACAGATCCAAAAACAACCCTAACTTATACAACAGATCACATCT
GGTTCTCTGATAAAAGAAGTTGCAGACCAATACCCGAAATTTTGTTTAGCCACAGAAGCA
ATAAAAATGTTCGAATATTTGAAATAGATGAAGGAAAGAGATGTTATACTTTGCCAACAA
TTAAGGATCAAGTATATTTGATAAGGGGTGTATTTCCCTTTGATAGTTTAAATTCTTCGT
TTTATGTTTATATCGGGGTAACAGAACTAGGTGAATTAAGATCGTCTAGACTCGAGGACT
TGGAAATCGAGGGAGTTTTTAGAGCCACCAAAGACTATATTGATTTCTGCTTATTGAAGG
AAGATGTCAATCCCTTCATTTCTCAGATTGAATTGAGGCCATTACCTGAAGAATACCTAC
ATGGTTTCGCTACTAGTGTTTTAAAACTGATAAGCAGAAATAATCTTGGTGACACAAATG
ATGATATAAGGTTCCCAGATGACCAAAATGATAGAATCTGGAAACGGAAAGCAACTTCAA
CTCCATCATCTGCCCTTCCCCTGTCTTCCAATGTCAGCAATGTTGACCTCAAAGACAGTG
TCACACCTCCTCTACAAGTCCTACAAACAGCTCTTACTCACCCTGAGCGATTGGAGTTCG
TCCATGATGGCCTCGAGACCGATGATTATGAATACTCTGTGTTTCTCCACTTTCTTGAAC
TAAATGGCACTGTCAGAGCAGGACAAAGGGTGTTTGACATCTATCTAAACAATGAGATTA
AAAAGGAGAAGTTTGATGTTTTGGCTGGAGGGTCCAAGAACAGTTACACTGCCTTGAACA
TTTCAGCAAATGGATCACTCAATATAACCTTAGTCAAGGCATCTGGATCTGAGTTTGGAC
CCCTTTTGAATGCCTATGAAATCCTGCAGGCACGGTCGTGGATTGAAGAGACCAACCAAA
AAGATTTGGAACTTATTCAGAAGATGAGAGAAGAACTGCTGCTGCACAACCGAGAAAATG
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AGGGAGCAATTCCTTACTTTGTCACTAAGATGACCAATTTACAAATACTGAACCTGAGCC
ACAACCAGTTCGATTCGTTATTCCCCTCGTTTCCACCGTCCTCCTTGCTGATATCATTGG

ATCTGAGCTACAATGATCTTGATGGACGGCTTCCAGAATCCATTATCTCACTGCCACATT
TAAAATCATTATATTTTGGCTGCAATCCATATATGAAGGACGAAGATACAACAAAGTTGA
ACAGTTCACTAATCAATACAGATTATGGGAGATGCAAAGGAAAAAAACCAAAGTTTGGAC
AAGTATTCGTGATTGGAGCTATTACAAGGGGATCACTTTTGATTACTTTGGCTGTTGGAA
TTCTATTTTTTTGCCGTTATAGACACAAGTCAATTACTTTGGAAGGATTTGGTGGAAAGA
CCTACCCAATGGCAACAAATATAATCTTCTCTTTGCCAAGCAAAGACGATTTCTTCATAA
AGTCTGTATCAGTTAAACCATTCACTTTGGAGTATATAGAGCAGGCTACAGAACAGTACA
AAACTTTGATAGGTGAAGGAGGATTTGGTTCTGTTTACAGAGGCACTCTAGACGATGGTC
AAGAAGTGGCAGTGAAAGTGCGGTCATCCACATCAACTCAGGGAACCCNAGAATTTGATA
ATGAGCTAAACCTACTTTCAGCTATACAACATGAGAACCTGGTGCCTCTTCTGGGTTACT
GTAATGAGTATGATCAACAAATTCTCGTGTATCCATTCATGTCCAATGGCTCTTTGCTAG
ATAGACTATACGGGGAAGCATCAAAGAGAAAAATATTAGACTGGCCAACTAGACTCTCTA
TTGCTCTCGGTGCAGCTCGAGGTTTGGCATATCTTCACACATTTCCAGGACGTTCTGTAA
TACACAGGGACGTAAAATCGAGCAATATACTGCTGGATCAGAGCATGTGTGCTAAGGTTG
CAGATTTTGGTTTCTCAAAATACGCTCCTCAGGAAGGAGACAGTTATGTTTCCCTTGAAG
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CATTGTGGAGAGTTGTGGAAGTAGCACTGCAATGTCTAGAACCCTACTCAACATATAGGC
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TGACACACCCTCAACCGAGATAGTAAATGGGTCGATGGAATTCTTTTGATTTGTTTTTTA
TCATTGCTTTAGTAATATCCCATTTTAAATGGTAAAGGAGAAAAATACTACTTTTGATTG
TATTTTCATCCACTCTATGTTTCTTGAAACTGAATCTCTCTTGCTCAGCCCCAGTTTTTA
TGGGTGAAAAGAATAATTTGGGTCAAATGCAAGTGAAACCATATGGTGCATAATTTGAAA
GCCATATTATATCATTTGCTAAGTCCAAAGTAAAAATTTCACAAACTAGTTAGATTGCGA
TTTAGTCTATACACACTTCAACAGAGCTATATACACTAT

or a deletion product, recombinant form and variant thereof.

7. The recombinant form of any of the nucleotide sequences of claims 1 to 5 which comprise a functional promoter sequence.

8. Oligonucleotide which comprises at least the functional part of the nucleotide sequence of any of claims 1 to 7.

9. Oligonucleotide hybridizing to any of the nucleotide sequences of claims 1 to 8.

10. Antibody capable of detecting the polypeptide having NORK biological activity.

11. Antibody capable of detecting the polypeptide encoded by the DNA sequences according to any of claims 2 to 9.

12. Transgenic plant which comprises the nucleotide sequence of any of claims 1 to 9.

13. The transgenic plant according to claim 12 which is a crop plant.

14. The transgenic plant according to claim 12 which is tobacco plant.

15. Transformant cell which contains the nucleotide sequence according to any of claims 1 to 9.

16. The amino acid sequence according to Figure 17 and a functional fragment thereof having NORK activity.

17. The amino acid sequence according to Figure 12 and a functional fragment thereof having NORK activity.

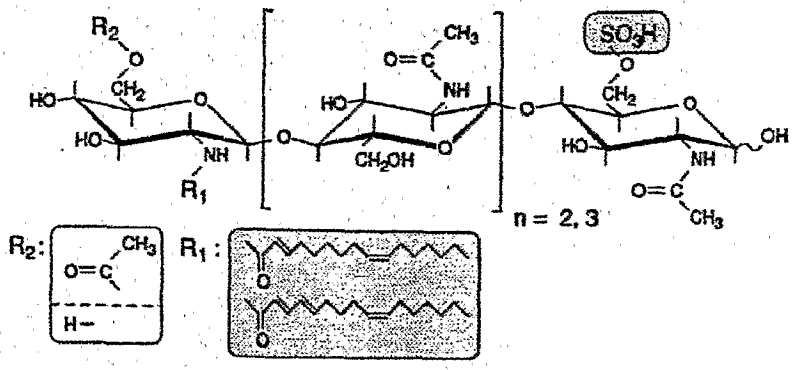
18. The amino acid sequence according to Figure 30 and a functional fragment thereof having NORK activity.

19. The amino acid sequence according to Figure 31 and a functional fragment thereof having NORK activity.

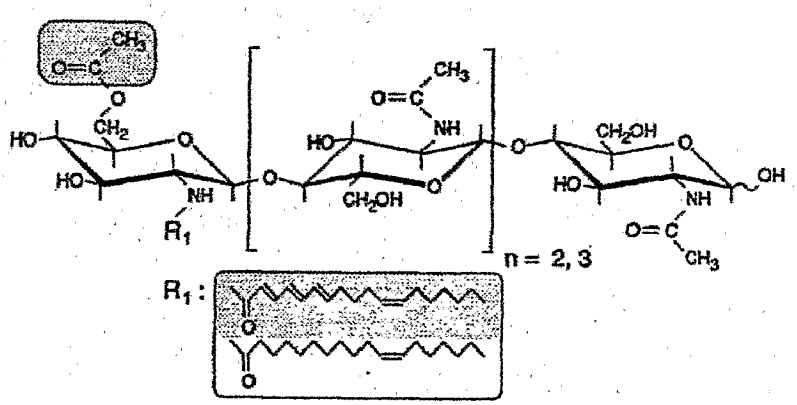
20. Process for preparing plants of Nod^+ phenotype from plants of Nod^- phenotype characterized by transforming a plant or the reproducing form thereof with a DNA according to any opf claims 1 to 9.

Figure 1. (1/2)

A.



B.



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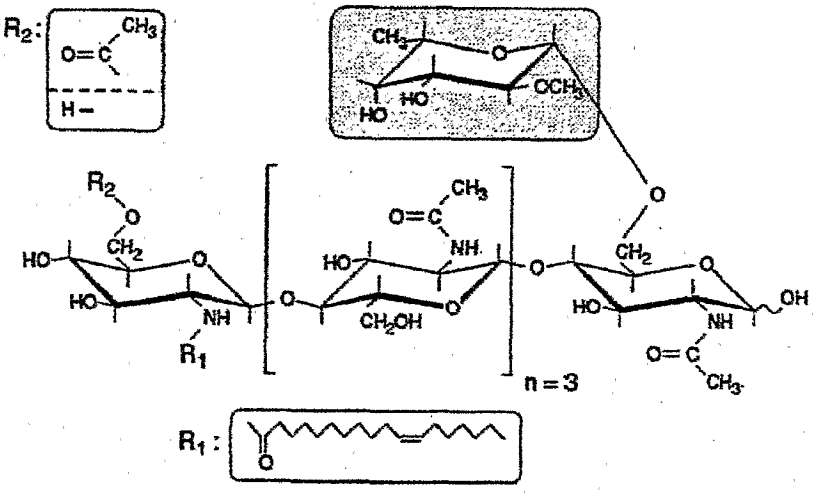
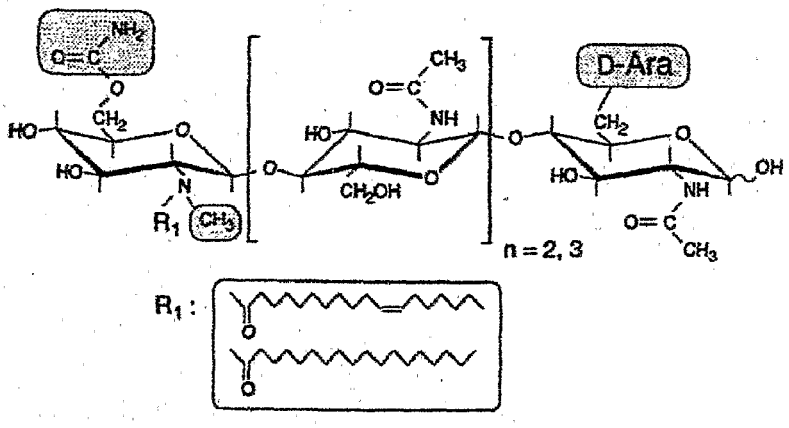


Figure 1. (cont. 2/2).

D.



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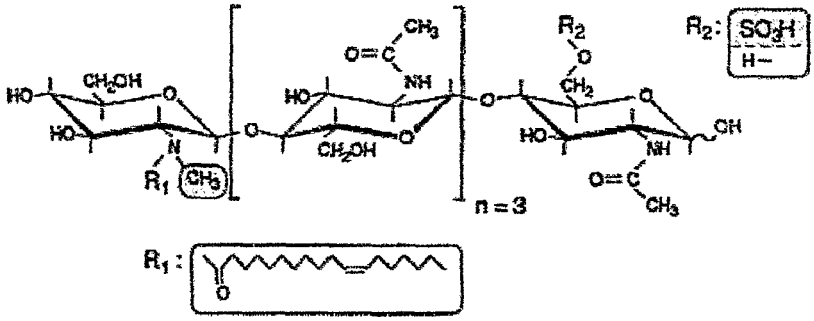

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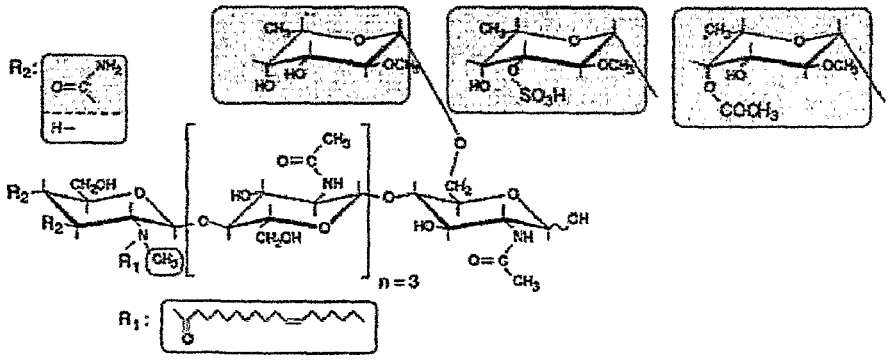
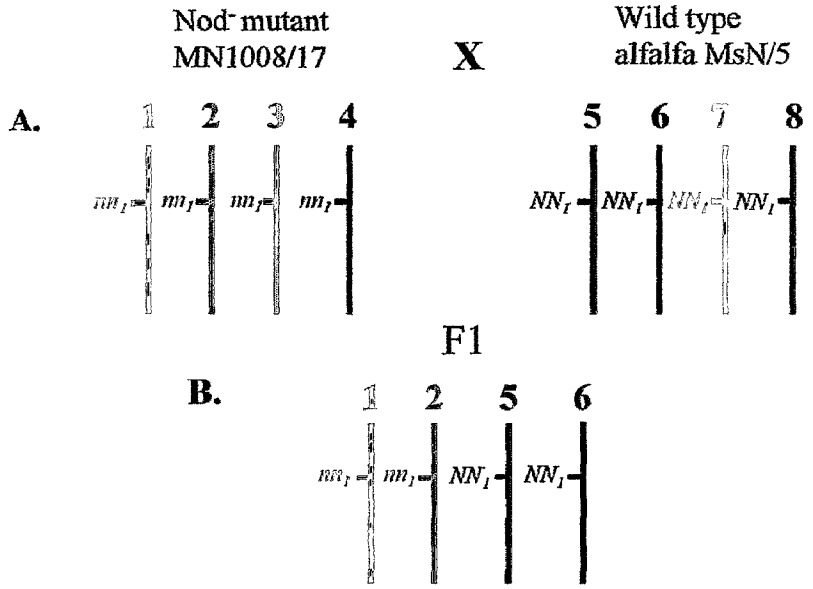


Figure 2.



C.

F1 gametes → / ↓	1 2 *m m*	1 5 *m NN*	1 6 *m NN*	2 5 *m NN*	2 6 *m NN*	5 6 *NN NN*
1 2 *m m*	1 1 2 2 *m m m m*	1 1 2 5 *m m m NN*	1 1 2 6 *m m m NN*	1 2 2 5 *m m m NN*	1 2 2 6 *m m m NN*	1 2 5 6 *m m NN NN*
1 5 *m NN*	1 1 2 5 *m m m NN*	1 1 5 5 *m m NN NN*	1 1 5 6 *m m NN NN*	1 2 5 5 *m m NN NN*	1 2 5 6 *m m NN NN*	1 5 5 6 *m NN NN NN*
1 6 *m NN*	1 1 2 6 *m m m NN*	1 1 5 6 *m m NN NN*	1 1 6 6 *m m NN NN*	1 2 5 6 *m m NN NN*	1 2 6 6 *m m NN NN*	1 5 6 6 *m NN NN NN*
2 5 *m NN*	1 2 2 5 *m m m NN*	1 2 5 5 *m m NN NN*	1 2 5 6 *m m NN NN*	2 2 5 5 *m m NN NN*	2 2 5 6 *m m NN NN*	2 5 5 6 *m NN NN NN*
2 6 *m NN*	1 2 2 6 *m m m NN*	1 2 5 6 *m m NN NN*	1 2 6 6 *m m NN NN*	2 2 5 6 *m m NN NN*	2 2 6 6 *m m NN NN*	2 5 6 6 *m NN NN NN*
5 6 *NN NN*	1 2 5 6 *m m NN NN*	1 5 5 6 *m NN NN NN*	1 5 6 6 *m NN NN NN*	2 5 5 6 *m NN NN NN*	2 5 6 6 *m NN NN NN*	5 5 6 6 *NN NN NN NN*

F2 population

For example: individual with genotype 1122: NAB 267; individual with genotype 5566: NAB 814

D.

The genotype of the homozygous wild type (Nod+) F3 individuals:

The genotype of the self-pollinated F2 individuals:

The genotype of the homozygous mutant (Nod-) F3 individuals:

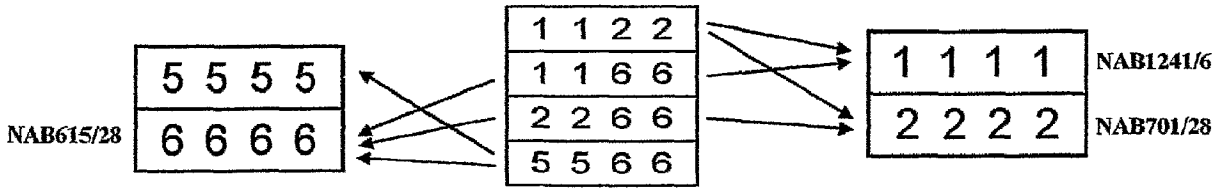


Figure 3.

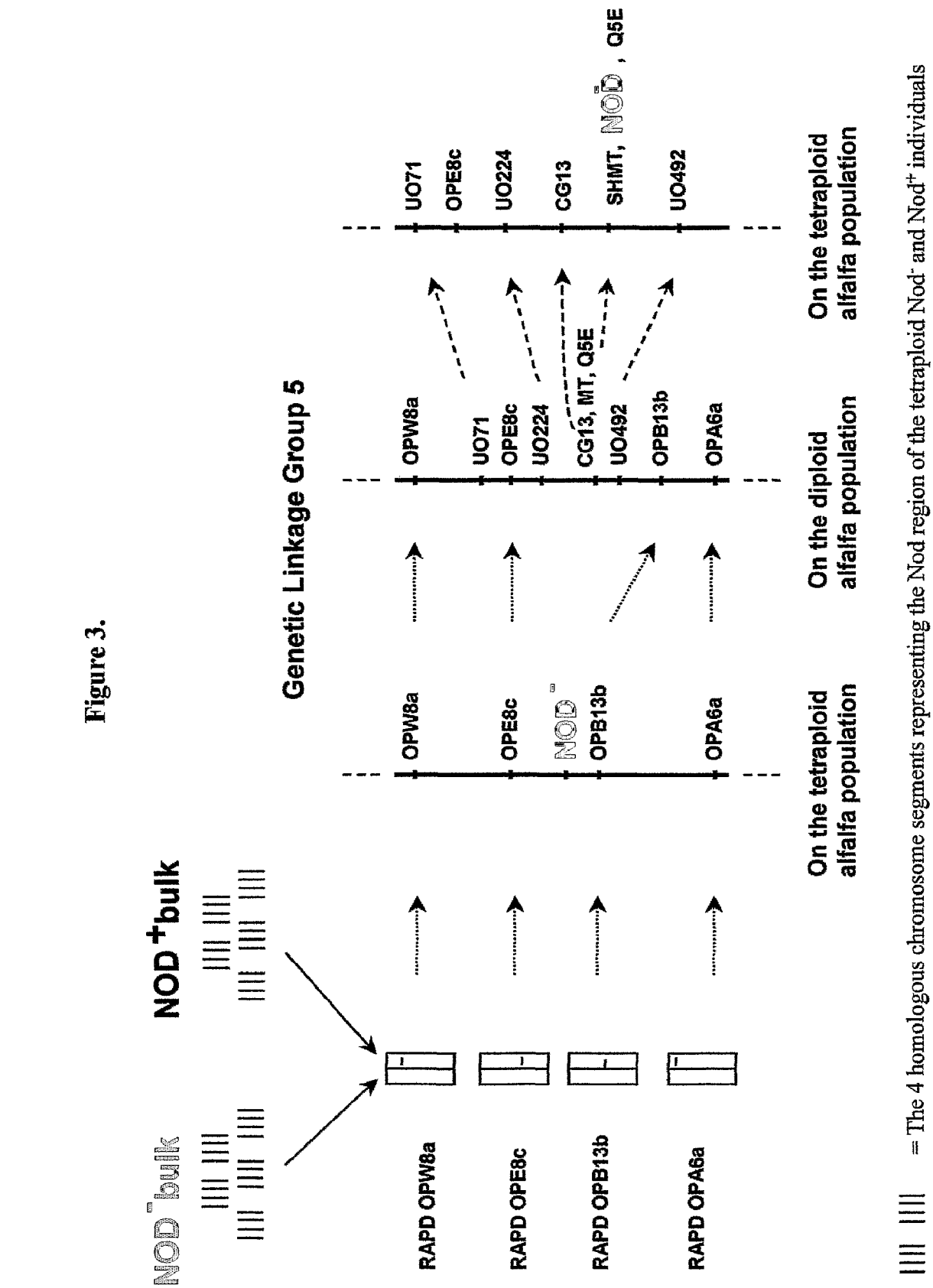
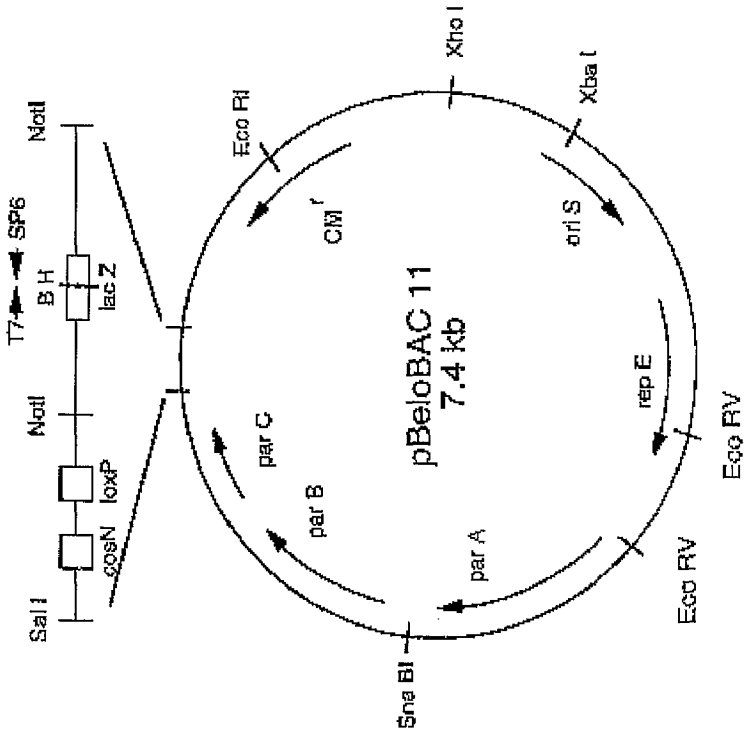

NOD⁻bulk
NOD⁺bulk
RAPD OPW8a
RAPD OPE8c
RAPD OPB13b
RAPD OPA6a
Genetic Linkage Group 5
OPW8a
OPE8c
NOD⁻
OPB13b
OPA6a
On the tetraploid alfalfa population
OPW8a
UO71
OPE8c
UO224
CG13, MT, Q5E
UO492
OPB13b
OPA6a
On the diploid alfalfa population
UO71
OPE8c
UO224
CG13
SHMT, NOD⁻, Q5E
UO492
On the tetraploid alfalfa population
= The 4 homologous chromosome segments representing the Nod region of the tetraploid Nod⁻ and Nod⁺ individuals

Figure 4.


pBeloBAC 11
7.4 kb
Eco RI
Xho I
Xba I
Eco RV
Sna BI
Eco RV
Sal I
NotI
NotI
T7
SP6
B H
lac Z
loxP
cosN
CM r
ori S
rep E
par C
par B
par A

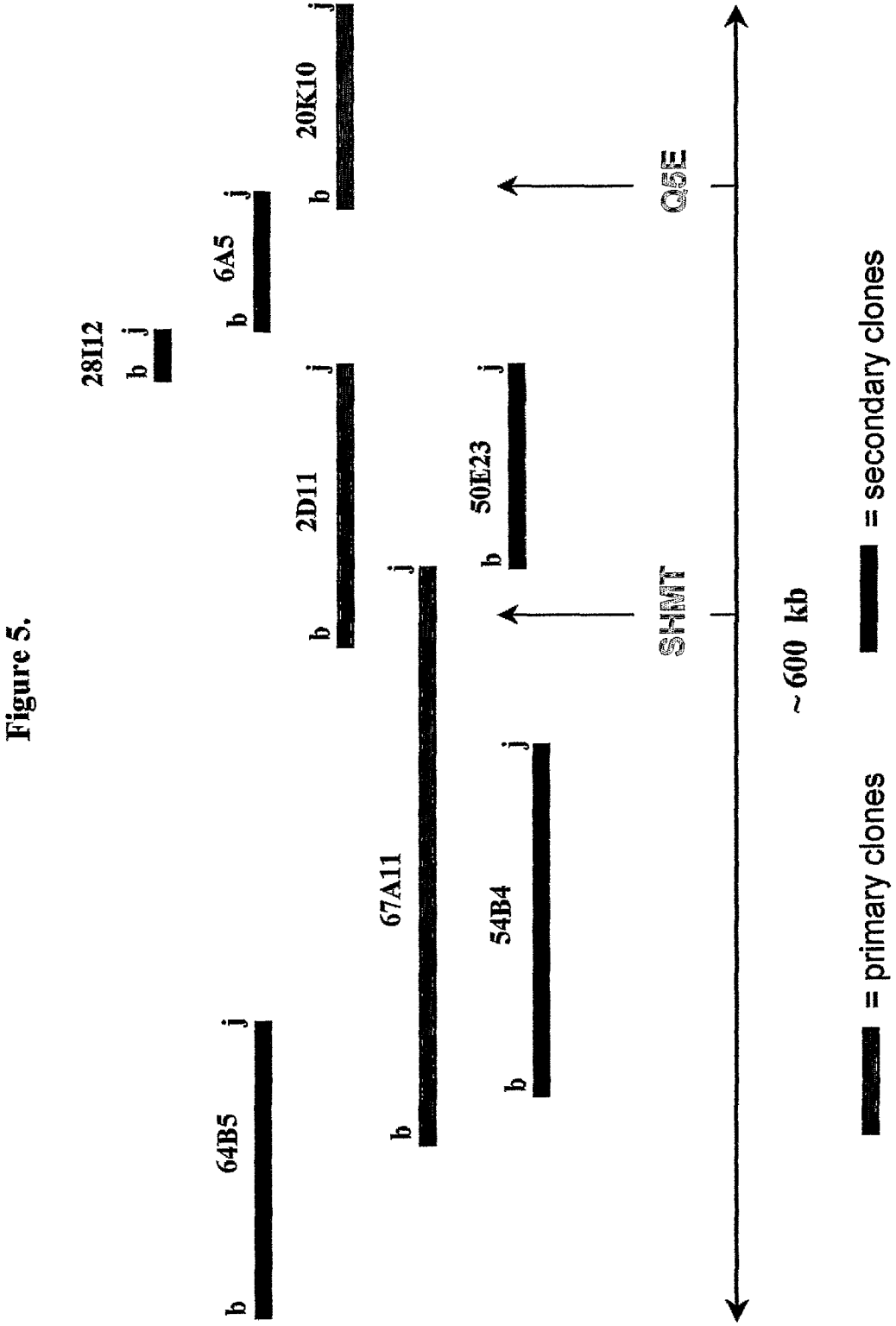
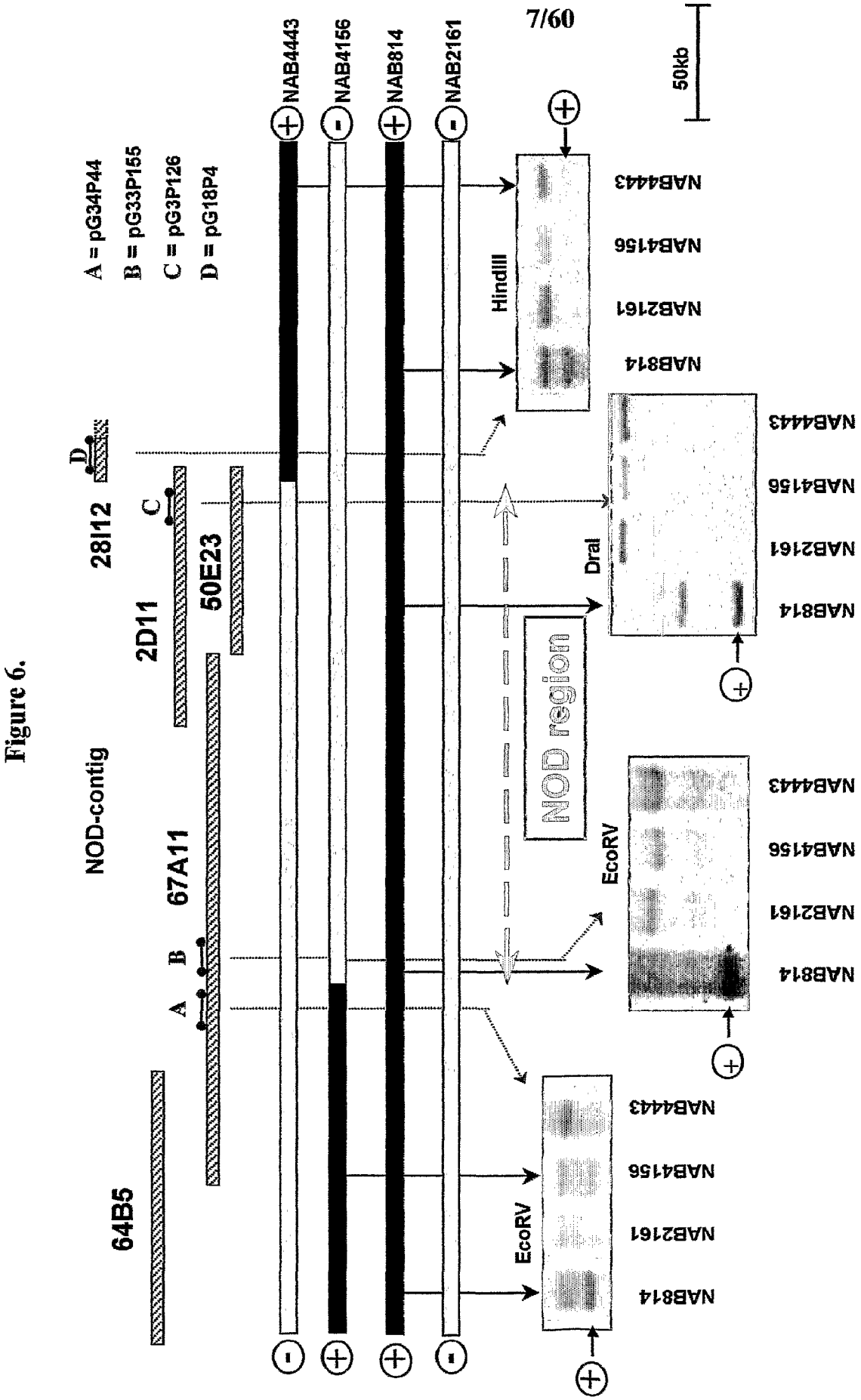

64B5
67A11
54B4
2D11
50E23
28I12
6A5
20K10
SHMT
Q5E
~ 600 kb
= primary clones
= secondary clones

Figure 5.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NAB4156
NAB814
NAB2161
50kb
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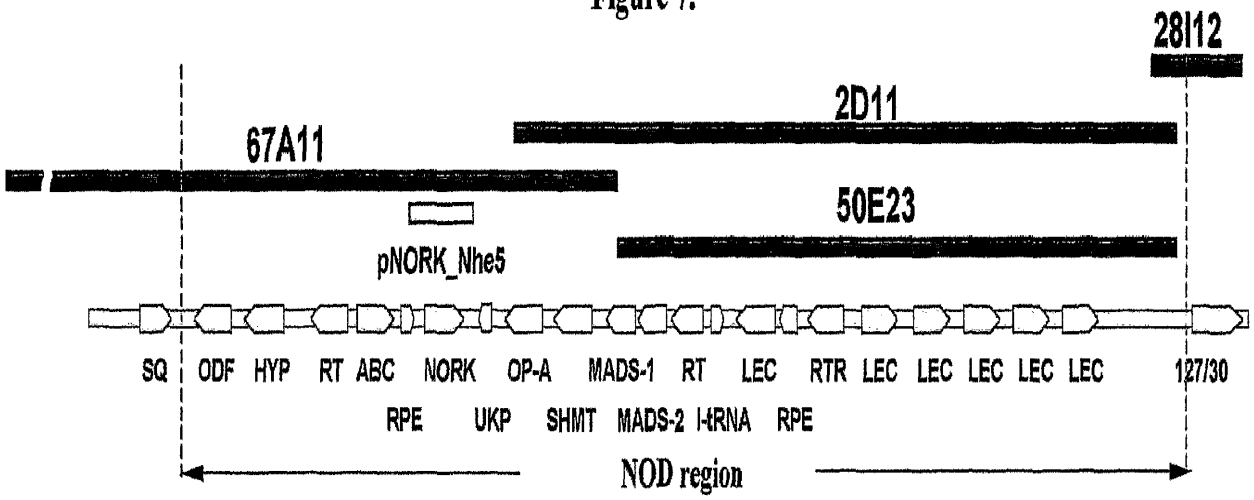
B = pG33P155
C = pG3P126
D = pG18P4
NOD-contig
64B5
67A11
2D11
28I12
50E23
HindIII
DraI
EcoRV
EcoRV
NOD region
NAB4443
NAB4156
NAB2161
NAB814

Figure 6.

Figure 7.



Abbrevation	Name of the gene	Accession number	BLAST score	E value
SQ:	Coding for squallen epoxidase	dbj/BAA24448.1	148	$2e^{-34}$
ODF:	Expressed in mouse sperm	gb/AAB54209.1	96.3	$2e^{-18}$
HYP:	Coding for hypothetical protein	gb/AAD15574.1	185	$3e^{-45}$
RT:	Coding for reverse transcriptase	emb/CAA73798.1	66	$7e^{-10}$
ABC:	Coding for ABC transporter	emb/CAB82705.1	131	$4e^{-29}$
RPE:	Repetitive DNA	gb/AAB50037.1	56.6	$6e^{-07}$
NORK:	Coding for receptor kinase (NORK)	dbj/BAB09508.1	82.3	$1e^{-13}$
UKP:	Coding for unknown protein	gb/AAD15572.1	88	$4e^{-17}$
OP-A:	Coding for oligopeptidáz A	dbj/BAA98181.1	208	$9e^{-62}$
SHMT:	Coding for serine hidroxymethyl transpherase	gb/AAA33687.1	139	$3e^{-34}$
MADS-1:	Coding for MADS-box protein	emb/CAA71739.1	51.5	$1e^{-05}$
MADS-2:	Coding for MADS-box protein	emb/CAA71739.1	51.5	$1e^{-05}$
RT:	Coding for reverse transcriptase	gb|AAD21515.1	34.8	6.2
I-tRNS:	Coding for isoleucine tRNA	dbj/D82067.1	129	$1e^{-28}$
LEC:	Coding for lectin precursor	gb/AAB51442.1	127	$1e^{-35}$
RPE:	Repetitive DNA	gb/AAB50037.1	122	$2e^{-26}$
RTR:	Retrotransposon	dbj|BAA96774.2|	201	$3e^{-50}$
LEC:	Coding for lectin precursor	gb/AAB51442.1	84.3	$1e^{-38}$
LEC:	Coding for lectin precursor	gb/AAG00508.1	96.7	$3e^{-36}$
LEC:	Coding for lectin precursor	gb/AAB51442.1	132	$5e^{-30}$
LEC:	Coding for lectin precursor	gb/AAB51442.1	137	$1e^{-31}$
LEC:	Coding for lectin precursor	gb/AAB51442.1	138	$8e^{-32}$
127/30:	LG127/30 like gene	emb/CAB37543.1	82.3	$7e^{-22}$

Figure 8. (1/3)

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Figure 8. (cont. 2/3)

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GGTCGTGGATTGAAGAGACCAACCAAAAAGATTGTAAGTGTACAATAAGATGCTAAATTG
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Figure 8. (cont. 3/3)

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TGACTGATAGTTCGGATGTTGCTAATGGAAATTGATAAGATGGTCAACATTGACTTAGTG
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ATTGTCTCTTGGTGATTGTTCAACAGACCCTCAACATTGCTCATCCTATCAGAACCCAAC
TTCCAATAGGCATTTGTTCTACTTAAAAGAAAATCGGAAGCTAGC

Figure 9.

A.

Forward primers:		Length (base)	Start point (bp)	Exon/Intron position
Pr_RKU5:	5'-AGGGGTTACTTGCTCTTTTGTGGT-3'	24	72	prom.
Pr_RKU6	5'-ATCTCTAACTCGTCTTCCATCTC-3'	23	847	prom.
Pr_RKU4	5'-TTCCAGGCCTAAAGTCAAACACCA-3'	24	897	E 1
Pr_RK-ATG	5'-TCTCTTTGGTACCATGATGGAGTTACAAGTTA-3'	32	1139	E 1
Pr_RKEx2U	5'-ATGGATCCGGGTTTGAGAGCATAGCATG-3'	28	1563	E 2
Pr_RKU3	5'-TGAGGGAGTTTTTAGAGCCACCAAAG-3'	26	1892	E 2
Pr_RKU2	5'-CACCCTGAGCGATTGGAGTTC-3'	21	2946	E 3
Pr_Pst-A5	5'-GAAATCCTGCAGGCACGGTCGTGG-3'	24	3222	E 3
Pr_RKU7	5'-AAGCATTGGAGAGTTGGAGTGG-3'	22	3426	E 4
Pr_RKU2X	5'-AAACAAGCACATGCAGATTAT-3'	21	4138	16
Pr_RK-ECO-5'	5'-GGCTGTTGGAATTCTATTTTTTTTGC-3'	25	4947	E 9
Pr_RKU1	5'-TACAGGGGCACTCTAGACGATGGT-3'	24	5679	E 10
Pr_Pst-B5	5'-AAGAGGAACTGCAGGGTATCTGG-3'	23	6956	E 13
Pr_RKU0	5'-GCAGAGGCATTGTGGAGAGT-3'	20	7631	E 15
Reverse primers:				
Pr_RKD5	5'-AGGGTGGTTTTTGGATCTGTGTAA-3'	24	1621	E 2
Pr_RKD4	5'-TTAAAACACTAGTACCGAAACC-3'	22	2028	E 2
Pr_RK_D3	5'-ACCCTCCAGCCAAAACATCAAA-3'	22	3120	E 3
Pr_Pst-A3	5'-ACGACCGTGCCTGCAGGATTTCA-3'	23	3246	E 3
Pr_RKD2x	5'-TAAATGTGGCAGTGAGATAATGGA-3'	24	4582	E 7
Pr_RK-ECO-3'	5'-GCAAAAAAATAGAATTCCAACAGCC-3'	25	4972	E 9
Pr_RKD2	5'-CCCTGAGTTGATGTGGATGAC-3'	21	5746	E 10
Pr_RKD7	5'-GATACCCTGCAGTTCCTCTTA-3'	21	6976	E 13
Pr_RKD1	5'-GGCCGATATGTTGAGTAGGGTTCT-3'	24	7698	E 15
Pr_RK-STOP	5'-AAAAGAGCTCCATCGACCCATTTACTATC-3'	29	7924	E 15
Pr_RKD0	5'-GAATTAGTCGCAAGTCAACC-3'	20	8236	E 15

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B.

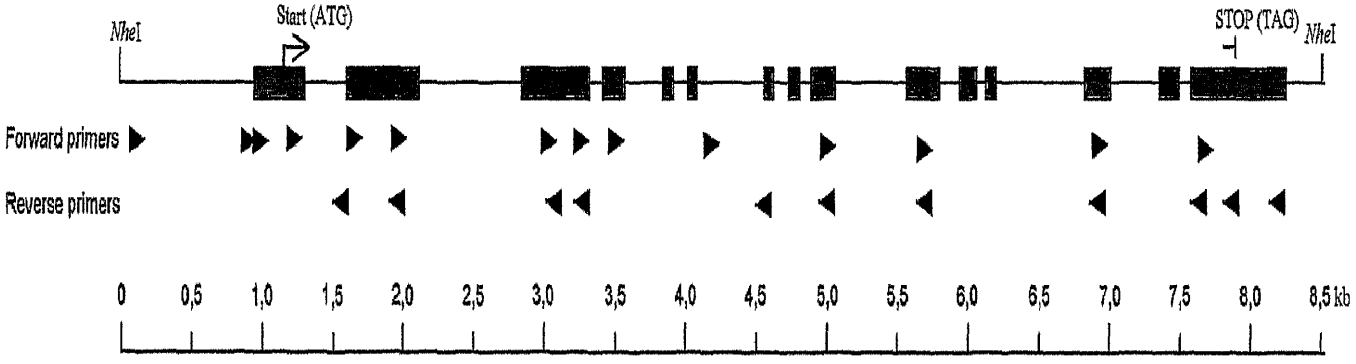

Figure 10.

TTCCAGGCCTAAAGTCAAACACCATATTTTAACAATATTCTTTCTTCTACAGCTTTTATA
CAAGTTCACTATATTATAGGATTGATCAGGGTTCATTTTTTCTTTCTTTGAAAAATCTCT
AAGGGGTGCTGTTTCCAAGGCAGAGAATGAAATAGAATTCAGAAGAATTTTTATGTTACT
ATAAAGGAAAGATGAAAAGTTAGTTAGCATGGATTCAAGTTTGATAACCCTATGGGGTAA
AATCTCTTTCAGATTATGATGGAGTTACAAGTTATTAGGATATTTAGATTGGTTGTGGCA
TTTGTTCTTTGTTTGTGTATATTTATCAGATCAGCTTCTTCTGCAACTAAAGGGTTTGAG
AGCATAGCATGTTGTGCTGATTCAAATTACACAGATCCAAAAACCACCCTAACTTATACA
ACAGATCACATCTGGTTCTCTGATAAAAGAAGTTGCAGACAAATACCCGAAATTTTGTTT
AGCCACAGAAGCAATAAAAATGTTCGAAAATTTGAAATATATGAAGGAAAGAGATGTTAT
AATTTGCCAACAGTTAAGGATCAAGTATATTTGATAAGGGGCATATTTCCCTTTGATAGT
TTAAATTCTTCGTTTTATGTTTCGATCGGGGTAACAGAACTAGGCGAATTAAGATCGTCT
AGGCTCGAGGACTTGGAAATTGAGGGAGTTTTTAGAGCCACCAAAGACTACATAGATTTC
TGCTTATTGAAGGAGGATGTCAATCCCTTCATTTCTCAGATTGAATTGAGGCCATTACCT
GAAGAATACCTACATGGTTTCGGTACTAGTGTTTTAAAACTGATAAGCAGAAACAATCTT
GGTGACACAAATGATGATATAAGGTTCCCAGATGACCAAAATGATAGAATCTGGAAACGG
AAAGAAACTTCAACTCCAACATCTGCCCTTCCACTGTCTTTCAATGTCAGCAATGTTGAC
CTCAAAGACAGTGTCACACCTCCTCTACAAGTCCTACAAACAGCTCTTACTCaCCCTGAG
CGATTGGAGTTCGTCCATGATGGCCTCGAGACCGATGATTATGAATACTCTGTGTTTCTC
CACTTTCTTGAACTAAATGGCACTGTCAGAGCAGGACAAAGGGTGTTTGACATCTATCTA
AACAATGAGATTAAAAAGGAGAAATTTGATGTTTTGGCTGGAGGGTCCAAGAACAGTTAC
ACTGCCTTGAACATTTCAGCAAATGGATCACTCAATATAACCTTAGTCAAGGCATCTGGA
TCTGAGTTTGGACCCCTTTTGAATGCCTATGAAATCCTGCAGGCACGGTCGTGGATTGAA
GAGACCAACCAAAAAGATTTGGAAGTTATTCAGAAGATGAGAGAAGAACTGCTGCTGCAC
AACCAAGAAAATGAAGCATTGGAGAGTTGGAGTGGAGACCCTTGTATGATTTTCCCCTGG
AAAGGAATAACATGTGATGATTCAACTGGTTCATCTATTATCACTAAGCTGGATCTTTCT
TCCAATAATCTCAAGGGAGCAATTCCTTCCATTGTCACTAAGATGACCAATTTACAAATA
CTGAACCTGAGCCACAACCAGTTCGATATGTTATTCCCCTCGTTTCCACCGTCCTCCTTG
CTGATATCATTGGATCTTAGCTACAATGATCTTTCAGGATGGCTTCCAGAATCCATTATC
TCACTGCCACATTTAAAATCATTATATTTTGGCTGCAATCCATCTATGAGTGACGAAGAT
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CCAAAGTTTGGACAAGTATTCGTGATTGGAGCTATTACAAGTGGATCACTTTTGATTACT
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TTCTTCATAAAGTCTGTATCAGTTAAACCGTTCACTTTGGAGTATATAGAGCAGGCTACA
GAACAGTACAAAACTTTAATAGGTGAAGGAGGATTTGGCTCTGTTTACAGGGGCACTCTA
GACGATGGTCAAGAAGTGGCAGTGAAAGTGCGGTCATCCACATCAACTCAGGGAACCCGA
GAATTTGATAATGAGCTAAACCTACTTTCAGCTATACAACATGAGAACCTGGTGCCTCTT
CTGGGTTACTGTAATGAGTATGATCAACAAATTCTCGTGTATCCTTTCATGTCCAATGGC
TCTTTGCTAGATAGACTATACGGGGAAGCATCAAAGAGAAAAATATTAGACTGGCCAACT
AGACTCTCTATTGCTCTCGGTGCAGCTCGAGGTTTGGCATATCTTCACACATTTCCAGGA
CGTTCTGTAATACACAGGGACGTAAAATCGAGCAATATACTGCTGGATCAGAGCATGTGT
GCTAAGGTTGCAGATTTTGGTTTCTCAAAATATGCTCCTCAGGAAGGAGACAGTTATGTT
TCCCTTGAAGTAAGAGGAACTGCAGGGTATCTGGATCCTGAGTACTACAAAACCCAGCAA
TTATCTGAAAAAAGTGATGTTTTCAGCTTTGGTGTGGTTCTTCTTGAAATTGTAAGCGGA
CGGGAACCTCTCAACATAAAGAGACCAAGGATCGAGTGGAGCTTGGTTGAATGGGCTAAA
CCATACATAAGAGCATCAAAGGTGGATGAAATTGTAGATCCTGGCATCAAGGGAGGATAT
CATGCAGAGGCATTGTGGAGAGTTGTGGAAGTAGCACTACAATGTCTAGAACCCTACTCA
ACATATCGGCCATGCATGGTTGATATTGTCCGCGAGTTGGAGGATGCTCTCATTATTGAA
AACAATGCATCTGAATACATGAAATCCATAGACAGCCTTGGAGGATCCAACCGCTACTCA
ATTGTTATGGACAAACGGGCGCTGCCTTCAACTACATCTACAGCAGAATCAACTATCACA
ACCCAAACCTTGTCACACCCTCAACCGAGATAGTAAATGGGTCGATGGAATTCTTTTGAT
TTGTTTTTGATCATTGCTTTAGTAATATCACATTTTAAATGGTAAAGGAGAAAAATACTA
CTTCTGATTGTATTTCCATCCACTCTATGTTTCTTGAAACTGAATCTCTCTTGCTCAGCC
CCAGTTTTTATGGGTGAAAAGAATAATTTGGGTCAAATGCAAGTGAAACCATATGGTGCA
TAATTTAAAAGCCATATCATATCATTTGCCAAGTCCAAAGTAAAAATTTCACAAACTAGT
TAGATTGCGATTTAGTCTATAGACACTTCAACAGAGCTATATACACTATGGTTGACTTGC
GA

14/60

Figure 11. (1/5)

NheI

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GCTAGCTTAATTTGTGAAGTAGTTTTCAGTCAGCTCATGCTGAACAGTTATGGTAGTTTT  BAC A17
ACAAGGATGTAAGGGGTTACTTGCTCTTTTGTGGTATCATTGAGCCACTTTCCACTTTCT  BAC A17
TAATTCTTTGCTATCAAATTTAAATATATTATATTATTGTAGGAAATATGAAAAAGAAAC  BAC A17
CCTAKATGCTTTAATTGTGCTAAGCATATAAGATACAAAAAAAAAAAAAGTCTAATCTCTT  BAC A17
TTGATAGGAAACTGACACAAGGTTCGATCCTCAGAACCAACTATGAGACTAATTTTTGCT  BAC A17
TGAATTGGTTAATCAACTGCATCGCACTCATTGAATAAAAAAGTACGTTTAGATTGACGG  BAC A17
TGAATTTAACATAATCACAGTTGACCACTTTGATTTTCACGAAAGTTGCATGTAACTTTC  BAC A17
GTGCTTGAATATAAAGTCACGGTGCCACAATGGTTTTGTCAATATCGCCGKGAATCCAAA  BAC A17
CATGCACTTAATATGTTTTTATATTAATGGAAACCTATGTATATAAATATAAAATACTTA  BAC A17
AGCATATATATCCTTGCAACAATACCAAAAAAGTTTAAAATTTGCTCCAAAGATACTATT  BAC A17
CACGGTTTCTATTGTGATGCATCCATGGAATGAGAAGGAAGAAAACAGAAGAAGACGAAG  BAC A17
GACTGGTCTGTCCAATTATTTAAACCATATGAATATTTTTATATACAGATAATGAAGCAA  BAC A17
CCACCACTAAAACAATAAGAATAAATAATAATGTTCAACATCATCAATAATAAGAATAAT  BAC A17
AATGTTTAACATCAACAATAAAACTATAGGAAAAAAACATAATCAACTATGCATTGTACT  BAC A17
AATTCAATCTCTAACTCGTCTTCCATCTCTTTCCTAGCTACCTCCTGCAGTTTCCT      BAC A17

TTCCAGGCCTAAAGTCAAACACCATATTTTAACAATATTCTTTCTTCTACAGCTTTTATA  BAC A17
TTCCAGGCCTAAAGTCAAACACCATATTTTAACAATATTCTTTCTTCTACAGCTTTTATA  cDNS A17

CAAGTTCACTATATTATAGGATTGATCAGGGTTCATTTTTTCTTTCTTTGAAAAATCTCT  BAC A17
CAAGTTCACTATATTATAGGATTGATCAGGGTTCATTTTTTCTTTCTTTGAAAAATCTCT  cDNS A17

AAGGGGTGCTGTTTCCAAGGCAGAGAATGAAATAGAATTCAGAAGAATTTTTATGTTACT  BAC A17
AAGGGGTGCTGTTTCCAAGGCAGAGAATGAAATAGAATTCAGAAGAATTTTTATGTTACT  cDNS A17

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AATCTCTTTCAGATTATGATGGAGTTACAAGTTATTAGGATATTTAGATTGGTTGTGGCA  cDNS A17

TTTGTTCTTTGTTTGTGTATATTTATCAGATCAGCTTCTTCTGCAACTAAAGGTTAGTAG  BAC A17
TTTGTTCTTTGTTTGTGTATATTTATCAGATCAGCTTCTTCTGCAACTAAAG          cDNS A17

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AACATAGAAAACAACATATTTTAATTAACATGAAAGGCCATGGGATGATCATAATTAATA
ATGAGAAGAAAATAAATAAGAAACTTGTTTTTTTTTACAAGGATTGTAAAATAGAACTAGT  I1
AGTTTCATACTTTCAATACTGAGAATCTTGAAACAATTTCACTTTTTCTTTATGTTGCTA
GAATTTCTTTCAAGGGAAAATCCAATTTTGTACAAAATGAATTTAACTTGTGACATTTTC
CTTGTAG

GGTTTGAGAGCATAGCATGTTGTGCTGATTCAAATTACACAGATCCAAAAACCACCCTAA  BAC A17
GGTTTGAGAGCATAGCATGTTGTGCTGATTCAAATTACACAGATCCAAAAACCACCCTAA  cDNS A17

CTTATACAACAGATCACATCTGGTTCTCTGATAAAAGAAGTTGCAGACAAATACCCGAAA  BAC A17
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GATGTTATAATTTGCCAACAGTTAAGGATCAAGTATATTTGATAAGGGGCATATTTCCCT  cDNS A17

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TTGATAGTTTAAATTCTTCGTTTTATGTTTCGATCGGGGTAACAGAACTAGGCGAATTAA  cDNS A17

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TAGATTTCTGCTTATTGAAGGAGGATGTCAATCCCTTCATTTCTCAGATTGAATTGAGGC  cDNS A17
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15/60

Figure 11. (cont. 2/5)

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CATTACCTGAAGAATACCTACATGGTTTCGGTACTAGTGTTTTAAAACTGATAAGCAGAA   BAC A17
CATTACCTGAAGAATACCTACATGGTTTCGGTACTAGTGTTTTAAAACTGATAAGCAGAA   cDNS A17

ACAATCTTGGTGACACAAATGATGATATAAGGTATGTGATCTTACTTTATTTTTAGGTAG   BAC A17
ACAATCTTGGTGACACAAATGATGATATAAG                                cDNS A17

ATTCCACCTCTATTTTACAGGGAGTGTCTCTCAGGAAACCTAAAAGGCTTAGGGTTTGTC
ACTACTTGGTTCTTATGGAAGCATCATATGTTCATACTTAGTAGATATATATTGTATAAA
AATCAACATCTTTCTCCGTAAGGGATAGAAATTGCAATTCATGTTATGTTGAGCATATTT
AAACCAAAATAATTTGAGGAATATGATGCATGAACCTTTCTGCCAAATGCATGACATAAC
CTATGTTTCACTTTACATATAGAATTATAAGATGTGTTTACATCTTTATATTAACTTTGA
TCTTTATGATGCACTGAGATACACCTGAATTTAAATAACAAATGGAGAAGCAAGAATCTA   I2
GAACTCTACCATTCAATCCATTGTAATATGGAACTCACAAGAATTAACTATTGCCACTTC
AGATTAAATACCCTAAGAGTGTTTTTGTGGATGAGGGAATGTTTTGAGGGAATGTAATGT
TTTGAGGGAATTCCTCAAAACATTACATTACCTCAAAATATTCCCCCATCCAAACACACT
ATAAAGGATCCTAATCTAGTGTAGCCTTAGTTTTCTATTGAAGTGATTTGTGTTTAGAGT
TTGGTCTGCATCTGCAACTTTGTTTTGTTGCTGTAATGCCATGACTATGAACACTGACAG
AAAGGTCTCATAATATCGGTATCTATCAATTTTAG

GTTCCCAGATGACCAAAATGATAGAATCTGGAAACGGAAAGAAACTTCAACTCCAACATC   BAC A17
GTTCCCAGATGACCAAAATGATAGAATCTGGAAACGGAAAGAAACTTCAACTCCAACATC   cDNS A17

TGCCCTTCCACTGTCTTTCAATGTCAGCAATGTTGACCTCAAAGACAGTGTCACACCTCC   BAC A17
TGCCCTTCCACTGTCTTTCAATGTCAGCAATGTTGACCTCAAAGACAGTGTCACACCTCC   cDNS A17

TCTACAAGTCCTACAAACAGCTCTTACTCACCCTGAGCGATTGGAGTTCGTCCATGATGG   BAC A17
TCTACAAGTCCTACAAACAGCTCTTACTCACCCTGAGCGATTGGAGTTCGTCCATGATGG   cDNS A17

CCTCGAGACCGATGATTATGAATACTCTGTGTTTCTCCACTTTCTTGAACTAAATGGCAC   BAC A17
CCTCGAGACCGATGATTATGAATACTCTGTGTTTCTCCACTTTCTTGAACTAAATGGCAC   cDNS A17

TGTCAGAGCAGGACAAAGGGTGTTTGACATCTATCTAAACAATGAGATTAAAAAGGAGAA   BAC A17
TGTCAGAGCAGGACAAAGGGTGTTTGACATCTATCTAAACAATGAGATTAAAAAGGAGAA   cDNS A17

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ATTTGATGTTTTGGCTGGAGGGTCCAAGAACAGTTACACTGCCTTGAACATTTCAGCAAA   cDNS A17

TGGATCACTCAATATAACCTTAGTCAAGGCATCTGGATCTGAGTTTGGACCCCTTTTGAA   BAC A17
TGGATCACTCAATATAACCTTAGTCAAGGCATCTGGATCTGAGTTTGGACCCCTTTTGAA   cDNS A17

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TGCCTATGAAATCCTGCAGGCACGGTCGTGGATTGAAGAGACCAACCAAAAAGATT       cDNS A17

GTGTACAACTAAGATTGCTAAATNGATGAATTTTATTAACCTTTAACCAATTTTCTATTT   I3
CATTCTCCCTCTTACACTAACACTTTTTTTCCTTTCAG

TGGAAGTTATTCAGAAGATGAGAGAAGAACTGCTGCTGCACAACCAAGAAAATGAAGCAT   BAC A17
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TGGAGAGTTGGAGTGGAGACCCTTGTATGATTTTCCCCTGGAAAGGAATAACATGTGATG   cDNS A17

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ATTCAACTGGTTCATCTATTATCACTAAGCT                                 cDNS A17
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16/60

Figure 11. (cont. 3/5)

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TAGATTTGCTTATTTGAGTTTATCTATTGAAATAAACACTTATGACACTGTTTGGAAGAG
CTTATGAAAACAACTTATAGTTTATACGAAAACAAGTTGACTTTGTTATATCTATTTTAT   I4
AGAAATAGCTTATAAGTAAGAACTTATATGATAAGCGTTTATGCTATAAACGCTCAATTT
AACTGTTTATCCAAACAGGACTTTAATGCATCCATATGTGTTTACAAGTTTTGCTCTATT
TTCTGCAG

GGATCTTTCTTCCAATAATCTCAAGGGAGCAATTCCTTCCATTGTCACTAAGATGACCAA  BAC A17
GGATCTTTCTTCCAATAATCTCAAGGGAGCAATTCCTTCCATTGTCACTAAGATGACCAA  cDNS A17

TTTACAAATACTGTACTGCTTCTAAATCTCATTTAAAAACATCCACATTATTTTTGTGGG  BAC A17
TTTACAAATACT                                                   cDNS A17

AACAGTGGACTGTTTTTATCCATTAGTTAATAATGTTGTAACATTTTTGTTATGCGCAG   I5

GAACCTGAGCCACAACCAGTTCGATATGTTATTCCCCTCGTTTCCACCGTCCTCCTTGCT  BAC A17
GAACCTGAGCCACAACCAGTTCGATATGTTATTCCCCTCGTTTCCACCGTCCTCCTTGCT  cDNS A17

GATATCATTGTAATTATCTCTTCTCATGTTTAACAAATGAACTAGGATGATACTAATATG  BAC A17
GATATCATT                                                      cDNS A17

AGCTTATAGCACTTCTATTATCCTTGTAAATGTTAACATAAACAAGCACATGCAGATTAT
AGAACTAAAATATGATAGAATATGCTGTATATAGTCCCAACTCATGTTGGTTACATAAGG
TAGATATTAGACAGTCTCAATGCTGAAACCAATATCTGTGTGCATGATGCATCTTAATCT
TTAAAAGATTTTATGATTAAGGCTGTTCTCTATGGCACTTCAGTAGGAGCAGTAACACAT  I6
CTATGTGAAAGTTCCATATGAAATCCTTGAGAAATATGTTTTGACATTATGTTTCATATA
TTGCTGAATTTCTTTCTTCACCAAGGTTCGAATCTTAGAGAAATTTCCTACAGTTATTAG
TGCCATTGAAAAATTACTACTAAATCTTTTTCAAATGATTGATCTACATTTATGGATCAG

GGATCTTAGCTACAATGATCTTTCAGGATGGCTTCCAGAATCCATTATCTCACTGCCACA  BAC A17
GGATCTTAGCTACAATGATCTTTCAGGATGGCTTCCAGAATCCATTATCTCACTGCCACA  cDNS A17

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TTTAAAATCATT                                                   cDNS A17

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CAG

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AATCAATACAGAGTATGAAGTATTATTGATGCATAACAATAGAAGTTTTTAAAATAAAAT  BAC A17
AATCAATACAGA                                                   cDNS A17

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ACACAAGTCAATTACTTTGGAAGGATTTGGAAAGACCTACCCAATGGCAACAA         cDNS A17

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TTCAACACAATGATAAAATAAGAGAAATATATTTAAAAAAACTAACATCCAGTGTGAATT  I9
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TGAAAAAAGAATACTTATGAACATGATAGATGATATTAGTTTCTGTAGAGGATCATTACT
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17/60

Figure 11. (cont. 4/5)

ATGATGGAAGTGATGATTTTAGCATGTTTGGTTTGGTCTAATCTACATTGTATATATGAC ATTCTCCAACAAAAACATGCAG	
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ATATAATTTTCTCTTTGCCAAGCAAAGACGATTTCTTCATAAAGTCTGTATCAGTTAAAC	cDNS A17
CGTTCACTTTGGAGTATATAGAGCAGGCTACAGAACAGTACAAAACTTTAATAGGTGAAG	BAC A17
CGTTCACTTTGGAGTATATAGAGCAGGCTACAGAACAGTACAAAACTTTAATAGGTGAAG	cDNS A17
GAGGATTTGGCTCTGTTTACAGGGGCACTCTAGACGATGGTCAAGAAGTGGCAGTGAAAG	BAC A17
GAGGATTTGGCTCTGTTTACAGGGGCACTCTAGACGATGGTCAAGAAGTGGCAGTGAAAG	cDNS A17
TGCGGTCATCCACATCAACTCAGGGAACCCGAGAATTTGATAATGAGGTATAATATGCAT	BAC A17
TGCGGTCATCCACATCAACTCAGGGAACCCGAGAATTTGATAATGAG	cDNS A17
TATCACTTTATTAGAGCAACAAGATTCCCACAGAATGTTCCTAATTAAAGCTTTGGCCAT ATATATAGTAGTTTAAGAATTTGTTCGGTTCCTATCACACCATCTGATTGGTCTATGATG GATCATGCAG	I10
CTAAACCTACTTTCAGCTATACAACATGAGAACCTGGTGCCTCTTCTGGGTTACTGTAAT	BAC A17
CTAAACCTACTTTCAGCTATACAACATGAGAACCTGGTGCCTCTTCTGGGTTACTGTAAT	cDNS A17
GAGTATGATCAACAAATTCTCGTGTATCCTTTCATGTCCAATGGCTCTTTGCTAGATAGA	BAC A17
GAGTATGATCAACAAATTCTCGTGTATCCTTTCATGTCCAATGGCTCTTTGCTAGATAGA	cDNS A17
CTATACGGTAAATATCTCACAATTCTTTTAGCAGATATGTTTTAATACACAGTAATTCTG	BAC A17
CTATACG	cDNS A17
AAATTTAATTTCATAACAG	I11
GGGAAGCATCAAAGAGAAAAATATTAGACTGGCCAACTAGACTCTCTATTGCTCTCGGTG	BAC A17
GGGAAGCATCAAAGAGAAAAATATTAGACTGGCCAACTAGACTCTCTATTGCTCTCGGTG	cDNS A17
CAGCTCGAGGCAAGTACCATGATGTTGTTTTACTTTTACTAAGTTGTGCATATGTACTAA	BAC A17
CAGCTCGAG	cDNS A17
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GTTTGGCATATCTTCACACATTTCCAGGACGTTCTGTAATACACAGGGACGTAAAATCGA	BAC A17
GTTTGGCATATCTTCACACATTTCCAGGACGTTCTGTAATACACAGGGACGTAAAATCGA	cDNS A17
GCAATATACTGCTGGATCAGAGCATGTGTGCTAAGGTTGCAGATTTTGGTTTCTCAAAAT	BAC A17
GCAATATACTGCTGGATCAGAGCATGTGTGCTAAGGTTGCAGATTTTGGTTTCTCAAAAT	cDNS A17
ATGCTCCTCAGGAAGGAGACAGTTATGTTTCCCTTGAAGTAAGAGGAACTGCAGGGTATC	BAC A17
ATGCTCCTCAGGAAGGAGACAGTTATGTTTCCCTTGAAGTAAGAGGAACTGCAGGGTATC	cDNS A17
TGGATCCTGAGTAAGTGAACTTAAATCCGTCTTAATCTGAATGTCTCATACTGCTCTCTC	BAC A17
TGGATCCTGA	cDNS A17
ATCTATCACTTTCAGCAAAATATTCAATACATTTCCCACACACAAGTTTGTTGGAACCAT CAGAAAACTGAAGAAATGTTTCTGATTATATTCCTATTCTGAATCTGAAGTGTATATTAA	

18/60

Figure 11. (cont. 5/5)

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TTTTAAACATACAAAACTAAAGTTCCCATATGGTGACTACAAATGATAAGAGATATTTAT  I13
CCATGTGAATTTGAATGGGATGAAGAAGAATCTAACATACCTAGTCCCATTTGTTTCACA
TGTTACAGAGAGTAACCATGACATGAACAAATTTCACAATAGCTGACGTTTATCCATATG
CAG

GTACTACAAAACCCAGCAATTATCTGAAAAAAGTGATGTTTTCAGCTTTGGTGTGGTTCT  BAC A17
GTACTACAAAACCCAGCAATTATCTGAAAAAAGTGATGTTTTCAGCTTTGGTGTGGTTCT  cDNS A17

TCTTGAAATTGTAAGCGGACGGGAACCTCTCAACATAAAGAGACCAAGGATCGAGTGGAG  BAC A17
TCTTGAAATTGTAAGCGGACGGGAACCTCTCAACATAAAGAGACCAAGGATCGAGTGGAG  cDNS A17

CTTGGTTGAATGGGTATGATCCATGACCCCATTTTTTTTACAAATATAATTTTCAAGTGG  BAC A17
CTTGGTTGAATGG                                                  cDNS A17

TTCATATATTTTAGTAGGTACTAACACATAACTTTCATGCAG                     I14

GCTAAACCATACATAAGAGCATCAAAGGTGGATGAAATTGTAGATCCTGGCATCAAGGGA  BAC A17
GCTAAACCATACATAAGAGCATCAAAGGTGGATGAAATTGTAGATCCTGGCATCAAGGGA  cDNS A17

GGATATCATGCAGAGGCATTGTGGAGAGTTGTGGAAGTAGCACTACAATGTCTAGAACCC  BAC A17
GGATATCATGCAGAGGCATTGTGGAGAGTTGTGGAAGTAGCACTACAATGTCTAGAACCC  cDNS A17

TACTCAACATATCGGCCATGCATGGTTGATATTGTCCGCGAGTTGGAGGATGCTCTCATT  BAC A17
TACTCAACATATCGGCCATGCATGGTTGATATTGTCCGCGAGTTGGAGGATGCTCTCATT  cDNS A17

ATTGAAAACAATGCATCTGAATACATGAAATCCATAGACAGCCTTGGAGGATCCAACCGC  BAC A17
ATTGAAAACAATGCATCTGAATACATGAAATCCATAGACAGCCTTGGAGGATCCAACCGC  cDNS A17

TACTCAATTGTTATGGACAAACGGGCGCTGCCTTCAACTACATCTACAGCAGAATCAACT  BAC A17
TACTCAATTGTTATGGACAAACGGGCGCTGCCTTCAACTACATCTACAGCAGAATCAACT  cDNS A17

ATCACAACCCAAACCTTGTCACACCCTCAACCGAGATAGTAAATGGGTCGATGGAATTCT  BAC A17
ATCACAACCCAAACCTTGTCACACCCTCAACCGAGATAGTAAATGGGTCGATGGAATTCT  cDNS A17

TTTGATTTGTTTTTGATCATTGCTTTAGTAATATCACATTTTAAATGGTAAAGGAGAAAA  BAC A17
TTTGATTTGTTTTTGATCATTGCTTTAGTAATATCACATTTTAAATGGTAAAGGAGAAAA  cDNS A17

ATACTACTTCTGATTGTATTTCCATCCACTCTATGTTTCTTGAAACTGAATCTCTCTTGC  BAC A17
ATACTACTTCTGATTGTATTTCCATCCACTCTATGTTTCTTGAAACTGAATCTCTCTTGC  cDNS A17

TCAGCCCCAGTTTTTATGGGTGAAAAGAATAATTTGGGTCAAATGCAAGTGAAACCATAT  BAC A17
TCAGCCCCAGTTTTTATGGGTGAAAAGAATAATTTGGGTCAAATGCAAGTGAAACCATAT  cDNS A17

GGTGCATAATTTAAAAGCCATATCATATCATTTGCCAAGTCCAAAGTAAAAATTTCACAA  BAC A17
GGTGCATAATTTAAAAGCCATATCATATCATTTGCCAAGTCCAAAGTAAAAATTTCACAA  cDNS A17

ACTAGTTAGATTGCGATTTAGTCTATAGACACTTCAACAGAGCTATATACACTATGGTTG  BAC A17
ACTAGTTAGATTGCGATTTAGTCTATAGACACTTCAACAGAGCTATATACACTATGGTTG  cDNS A17

ACTTGCGACTAATTCGCTCAAGCAGGAGGAACACATATATATGGGAAACACTTGTAGAAC  BAC A17
ACTTGCGA                                                       cDNS A17

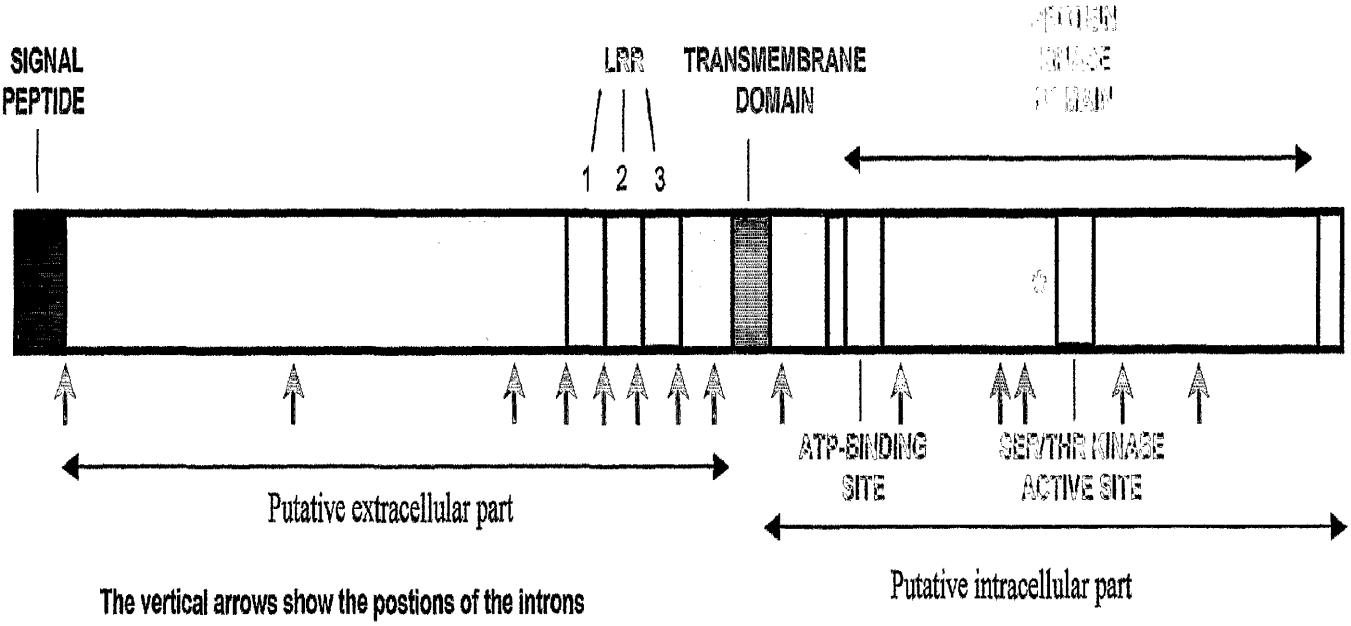
TATTTTGTTTATAGAAATGAAAATATTTTCCGTTTTTAATTTTTGTTTTCATTAAGTTTG  BAC A17
AATTATTTTTACTTTGGTTCATACATGATTCTAAATTATGTTTGAAGTTATGGTAAGAAA  BAC A17
GTTGTCATTGCGAAAAAATACGGCCGCAATTGCCGCTGTGCATCTTCAGCTCAAGTGATT  BAC A17
GTCTCTTGGTGATTGTTCAACAGACCCTCAaCATTGCTCATCCTATCAGAACCCAACTTC  BAC A17
CAATAGGCATTTGTTCTACTTAAAAGAAAATCGGAAGCTAGC                    BAC A17
                                        NheI
```

Figure 12.

MMELQVIRIFRLVVAFVLCLCIFIRSASSATKGFESIACCADSNYTDPKTTLTYTTDHIW
FSDKRSCRQIPEILFSHRSNKNVRKFEIYEGKRCYNLPTVKDQVYLIRGIFPFDSLNSSF
YVSIGVTELGELRSSRLEDLEIEGVFRATKDYIDFCLLKEDVNPFISQIELRPLPEEYLH
GFGTSVLKLISRNNLGDTNDDIRFPDDQNDRIWKRKETSTPTSALPLSFNVSNVDLKDSV
TPPLQVLQTALTHPERLEFVHDGLETDDYEYSVFLHFLELNGTVRAGQRVFDIYLNNEIK
KEKFDVLAGGSKNSYTALNISANGSLNITLVKASGSEFGPLLNAYEILQARSWIEETNQK
DLEVIQKMREELLLHNQENEALESWSGDPCMIFPWKGITCDDSTGSSIITKLDLSSNNLK
GAIPSIVTKMTNLQILNLSHNQFDMLFPSFPPSSLLISLDLSYNDLSGWLPESIISLPHL
KSLYFGCNPSMSDEDTTKLNSSLINTDYGRCKAKKPKFGQVFVIGAITSGSLLITLAVGI
LFFCRYRHKSITLEGFGKTYPMATNIIFSLPSKDDFFIKSVSVKPFTLEYIEQATEQYKT
LIGEGGFGSVYRGTLDDGQEVAVKVRSSTSTQGTREFDNELNLLSAIQHENLVPLLGYCN
EYDQQILVYPFMSNGSLLDRLYGEASKRKILDWPTRLSIALGAARGLAYLHTFPGRSVIH
RDVKSSNILLDQSMCAKVADFGFSKYAPQEGDSYVSLEVRGTAGYLDPEYYKTQQLSEKS
DVFSFGVVLLEIVSGREPLNIKRPRIEWSLVEWAKPYIRASKVDEIVDPGIKGGYHAEAL
WRVVEVALQCLEPYSTYRPCMVDIVRELEDALIIENNASEYMKSIDSLGGSNRYSIVMDK
RALPSTTSTAESTITTQTLSHPQPR*

Figure 13.

A.



The vertical arrows show the postions of the introns

LRR = Leucine Rich Repeats The star (*) indicates the site of the "stop codon" mutation inthe NORK gene of the MN1008 plant

B.

The name of the domain	Position within the protein
Signal peptide	1-28 aa
1. Leucine Rich Repeat (LRR)	405-429 aa
2. Leucine Rich Repeat (LRR)	430-454 aa
3. Leucine Rich Repeat (LRR)	455-476 aa
Transmembrane domain	523-543 aa
ATP binding site	601-623 aa
Serine-Threonine kinase active site	717-729 aa
Putative N-glycosylation sites	43-46; 116-119; 257-260; 318-320; 322-325; 326-329; 436-439; 499-502; 628-631; 673-676; 876-879 aa
Putative phosphorilation (autophosphorilation) sites	61-63; 65-67; 75-77; 78-80; 98-100; 133-135; 168-170; 173-175; 193-195; 222-224; 495-497; 581-583; 685-687; 776-778; 793-795; 855-857; 890-892 aa

21/60

Figure 14.

TTCCAGGCCTAAAGTCAAACACCATATTTTAACAATATTCTTTCTTCTACAAGGGTATAA
CTTTTATACAAGTTCACTATATTATAGGATTGATCAAGGTTCATTTTTTCTTTCTTTGAA
AAATCTCTAAGGGGTGCTGTTTCCAAGGCAGAAAATGAAATAGAATTCAGAAGAATTTTT
ATGGTACTAAAGGGAAGATGAGAAGTTAGTTAGCATGGATTCAAATTTGATAACCCTTTG
GGGTAAAATCTCTTTCAGATTATAATGGAGCTACAAGTTATTAGGATATTTAGATTGGTT
GTGGCATGTGTTCTTTGTTTGTGTATATTTATCAGATCAGCTTCTTCTGCAACTAAAGGG
TTTGAGAGCATAGCATGTTGTGCTGATTCCAATTACACAGATCCAAAAACCACCCTAACT
TATACAACAGATCACATCTGGTTCTCTGATAAAAGAAGTTGCAGACCAATACCCGAAATT
TTGTTTAGCCACAGAAGCAATAAAAATGTTCGAATATTTGAAATAGATGAAGGAAAGAGA
TGTTATACTTTGCCAACAATTAAGGATCAAGTATATTTGATAAGGGGTGTATTTCCCTTT
GATAGTTTAAATTCTTCGTTTTATGTTTATATCGGGGTAACAGAACTAGGTGAATTAAGA
TCGTCTAGACTCGAGGACTTGGAAATTGAGGGAGTTTTTAGAGCCACCAAAGACTATATT
GATTTCTGCTTATTGAAGGAGGATGTCAATCCCTTCATTTCTCAGATTGAATTGAGGCCA
TTACCTGAAGAATACCTACATGGTTTCGCTACTAGTGTTTTAAAACTGATAAGCAGAAAC
AATCTTGGTGACATAAATGATGATATCAGGTTCCCAGATGACCGAAATGATAGAATCTGG
AAACGGAAAGCAACTTCAACTCCATCATCTGCCCTTCCACTGTCTTTCAATGTCAGCAAT
GTTGACCTCAAAGACAGTGTCGCACCTCCTCTACAAGTCCTACAAACAGCTCTTACTCAC
CCTGAGCGATTGGAGTTTGTCCATGATGGCCTCGAGACCGATGATTATGAATACTCTGTG
TTTCTCCACTTTCTTGAACTAAATGGCACTGTCAGAGCAGGACAAAGGGTGTTTGACATC
TATCTAAACAATGAGATTAAAAAGGAGAAGTTTGATGTTTTGGCTGGAGGGTCCAAGAAC
AGTTACACTGCCTTGAACATTTCAGCAAATGGATCACTCAATATAACCTTAGTCAAGGCA
TCTGGATCTGAGTTTGGACCCCTTTTGAATGCCTATGAAATCCTGCAGGCACGGTCGTGG
ATTGAAGAGACCAACCAAAAAGATTTGGAACTTATTCAGAAGACAAGAGAAGAACTGCTG
CTGCACAACCAAGAAAATGAAGCATTGGAGAGTTGGAGTGGAGACCCTTGTATGATTTTC
CCCTGGAAAGGAATAACATGTGATGATTCAACTGGTTCATCTATTATCACTATGCTGGAT
CTTTCTTCCAATAATCTCAAGGGAGCAATTCCTTACTTTGTCACTAAGATGACCAATTTA
CAAATACTGAACCTGAGCCACAACCAGTTCGATTCGTTATTCCCCTCGTTTCCACCGTCC
TCCTTGCTGATATCATTGGATCTGAGCTACAATGATCTTGATGGACGGCTTCCAGAATCC
ATTATCTCACTGCCACATTTAAAATCATTATATTTTGGCTGCAATCCATATATGAAGGAC
GAAGATACAACAAAGTTGAACAGTTCACTAATCAATACAGATTATGGGAGATGCAAAGGA
AAAAAACCAAAGTTTGGACAAGTATTCGTGATTGGAGCTATTACAAGTGGATCACTTTTG
ATTACTTTGGCTGTTGGAATTCTATTTTTTTGCCGTTATAGACACAAGTCAATTACTTTG
GAAGGATTTGGTGGAAAGACCTACCCAATGGCAACAAATATAATCTTCTCTTTGCCAAGC
AAAGACGATTTCTTCATAAAGTCTGTATCAGTTAAACCATTCACTTTGGAGTATATAGAG
CAGGCTACAGAACAGTACAAAACTTTGATATGTGAAGGAGGATTTGGTTCTGTTTACAGA
GGCACTCTAGACGATGGTCAAGAAGTGGCAGTGAAAGTGCGGTCATCCACATCAACTCAG
GGAACCAAGGAATTTGATAACGAGCTAAACCTACTTTCAGCTATACAACATGAGAACCTG
GTGCCTCTTCTGGGTTACTGTAATGAGTATGATCAACAAATTCTCGTGTATCCATTCATG
TCTAATGGCTCTTTGCTAGATAGATTATACGGGGAAGCATCAAAGAGAAAAATATTAGAC
TGGCCAACTAGACTCTCTATTGCTCTCGGTGCACCTCGAGGTTTGGCATAGCTTCACACA
TTTCCAGGACCTTCTGTAATACACAGGGACCTAAAATCGAGCAATATACTGCTGGATCAG
AGCATGTGTGCTAAGGTTGCAGATTTTGGTTTCTCAAAATACGCTCCTCAGGAAGGAGAC
AGTTATGTTTCCCTTGAAGTAAGAGGAACTGCAGGGTATCTGGATCCTGAGTACTACAAA
ACCCAGCAATTATCTGAAAAAAGTGATGTTTTCAGCTTTGGTGTGGTTCTACTTGAAATT
GTAAGTGGACGGGAACCTCTCAACATAAAGAGACCACGGATCGAGTGGAGCTTGGTTGAA
TGGGCTAAACCATACATAAGAGCATCAAAGGTGGATGAAATTGTAGATCCTGGCATCAAG
GGAGGATATCATGCAGAAGCATTGTGGAGAGTTGTGGAAGTAGCACTGCAATGTCTAGAA
CCCTACTCAACATATAGGCCATGCATGGTTGATATTGTCCGCGAGTTGGAGGATGCTCTC
ATTATTGAAAACAATGCATCTGAATACATGAAATCCATAGACAGCCTTGGAGGATCCAAC
CGCTACTCAATTGTTATGGACAAACGGGCGCTGCCTTCAACTACATCTACAGCAGAATCA
ACTATCACAACCCAAACCTTGACACACCCTCAACCGAGATAGTAAATGGGTCGATGGAAT
TCTTTTGATTTGTTTTTGATCATTGCTTTAGTAATATCCCATTTTCAATGGTAAAGGAGA
AAAATACTACTTTTGATTGTATTTTCATCCACTCTATGTTTCTTGAAACTGAATCTCTCT
TGCTCAGCCCCAGTTTTTATGGGTGAAAAGAATAATTTGGGTCAAATGCAAGTGAAACCA
TATGGTGCATAATTTGAAAGCCATATTATATCATTTGCCAAGTCCAAAGTAAAAATTTCA
CAAACTAGTTAGATTGCGATTTAGTCTATAGACACTTCAACAGAGCTATATACACTATGG
TTGACTTGCGACTAATTC

22/60

Figure 15.

CCATATTTTAACAATATTCTTTCTTCTACAAGGGTATAACTTTTATACAAGTTCACTATA
TTATAGGATTGATCAAGGTTCATTTTTTCTTTCTTTGAAAAATCTCTAAGGGGTGTGGTT
TCCAAGGCAGAAAATGAAATAGAATGCAGAAGAATTTGTATGGTACTATAAAGGGAAGAT
GAAAAGTTAGTTAGCATGGATTCAAGTTTGATAACCCTTTGGGGTAAAATCTCTTTCAGA
TTATGATGGAGCTACAAGTTATTAGGATATTTAGATTGGTTGTGGCATGTGTTCTTTGTT
TGTGTATATTTATCAGATCAGCTTCTTCTGCAACTAAAGGGTTTGAGAGCATATCATGTT
GTGCTGATTCCAATTACACAGATCCAAAAACAACCCTAACTTATACAACAGATCACATCT
GGTTCTCTGATAAAAGAAGTTGCAGACCAATACCCGAAATTTTGTTTAGCCACAGAAGCA
ATAAAAATGTTCGAATATTTGAAATAGATGAAGGAAAGAGATGTTATACTTTGCCAACAA
TTAAGGATCAAGTATATTTGATAAGGGGTGTATTTCCCTTTGATAGTTTAAATTCTTCGT
TTTATGTTTATATCGGGGTAACAGAACTAGGTGAATTAAGATCGTCTAGACTCGAGGACT
TGGAAATCGAGGGAGTTTTTAGAGCCACCAAAGACTATATTGATTTCTGCTTATTGAAGG
AAGATGTCAATCCCTTCATTTCTCAGATTGAATTGAGGCCATTACCTGAAGAATACCTAC
ATGGTTTCGCTACTAGTGTTTTAAAACTGATAAGCAGAAATAATCTTGGTGACACAAATG
ATGATATAAGGTTCCCAGATGACCAAAATGATAGAATCTGGAAACGGAAAGCAACTTCAA
CTCCATCATCTGCCCTTCCCCTGTCTTCCAATGTCAGCAATGTTGACCTCAAAGACAGTG
TCACACCTCCTCTACAAGTCCTACAAACAGCTCTTACTCACCCTGAGCGATTGGAGTTCG
TCCATGATGGCCTCGAGACCGATGATTATGAATACTCTGTGTTTCTCCACTTTCTTGAAC
TAAATGGCACTGTCAGAGCAGGACAAAGGGTGTTTGACATCTATCTAAACAATGAGATTA
AAAAGGAGAAGTTTGATGTTTTGGCTGGAGGGTCCAAGAACAGTTACACTGCCTTGAACA
TTTCAGCAAATGGATCACTCAATATAACCTTAGTCAAGGCATCTGGATCTGAGTTTGGAC
CCCTTTTGAATGCCTATGAAATCCTGCAGGCACGGTCGTGGATTGAAGAGACCAACCAAA
AAGATTTGGAACTTATTCAGAAGATGAGAGAAGAACTGCTGCTGCACAACCGAGAAAATG
AAGCATTGGAGAGTTGGAGTGGAGACCCTTGTATGATTTTCCCCTGGAAAGGAATAACAT
GTGATGATTCAACTGGTTCATCTATTATCACTATGCTGGATCTTTCTTCCAATAATCTCA
AGGGAGCAATTCCTTACTTTGTCACTAAGATGACCAATTTACAAATACTGAACCTGAGCC
ACAACCAGTTCGATTCGTTATTCCCCTCGTTTCCACCGTCCTCCTTGCTGATATCATTGG
ATCTGAGCTACAATGATCTTGATGGACGGCTTCCAGAATCCATTATCTCACTGCCACATT
TAAAATCATTATATTTTGGCTGCAATCCATATATGAAGGACGAAGATACAACAAAGTTGA
ACAGTTCACTAATCAATACAGATTATGGGAGATGCAAAGGAAAAAAACCAAAGTTTGGAC
AAGTATTCGTGATTGGAGCTATTACAAGGGGATCACTTTTGATTACTTTGGCTGTTGGAA
TTCTATTTTTTTGCCGTTATAGACACAAGTCAATTACTTTGGAAGGATTTGGTGGAAAGA
CCTACCCAATGGCAACAAATATAATCTTCTCTTTGCCAAGCAAAGACGATTTCTTCATAA
AGTCTGTATCAGTTAAACCATTCACTTTGGAGTATATAGAGCAGGCTACAGAACAGTACA
AAACTTTGATAGGTGAAGGAGGATTTGGTTCTGTTTACAGAGGCACTCTAGACGATGGTC
AAGAAGTGGCAGTGAAAGTGCGGTCATCCACATCAACTCAGGGAACCCNAGAATTTGATA
ATGAGCTAAACCTACTTTCAGCTATACAACATGAGAACCTGGTGCCTCTTCTGGGTTACT
GTAATGAGTATGATCAACAAATTCTCGTGTATCCATTCATGTCCAATGGCTCTTTGCTAG
ATAGACTATACGGGGAAGCATCAAAGAGAAAAATATTAGACTGGCCAACTAGACTCTCTA
TTGCTCTCGGTGCAGCTCGAGGTTTGGCATATCTTCACACATTTCCAGGACGTTCTGTAA
TACACAGGGACGTAAAATCGAGCAATATACTGCTGGATCAGAGCATGTGTGCTAAGGTTG
CAGATTTTGGTTTCTCAAAATACGCTCCTCAGGAAGGAGACAGTTATGTTTCCCTTGAAG
TAAGAGGAACTGCAGGGTATCTGGATCCTGAGTACTACAAAACCCAGCAATTATCTGAAA
AAAGTGATGTTTTCAGCTTTGGTGTGGTTCTACTTGAAATTGTAAGTGGACGGGAACCTC
TCAACATAAAGAGACCACGGATCGAGTGGAGCTTGGTTGAATGGGCTAAACCATACATAA
GAGCATCAAAGGTGGATGAAATTGTAGATCCTGGCATCAAGGGAGGATATCATGCAGAAG
CATTGTGGAGAGTTGTGGAAGTAGCACTGCAATGTCTAGAACCCTACTCAACATATAGGC
CATGCATGGTTGATATTGTCCGCGAGTTGGAGGATGCTCTCATTATTGAAAACAATGCAT
CTGAATACATGAAATCCATAGACAGCCTTGGAGGATCCAACCGCTACTCAATTGTTATGG
ACAAACGGGCGCTGCCTTCAACTACATCTACAGCAGAATCAACTATCACAACCCAAACCT
TGACACACCCTCAACCGAGATAGTAAATGGGTCGATGGAATTCTTTTGATTTGTTTTTTA
TCATTGCTTTAGTAATATCCCATTTTAAATGGTAAAGGAGAAAAATACTACTTTTGATTG
TATTTTCATCCACTCTATGTTTCTTGAAACTGAATCTCTCTTGCTCAGCCCCAGTTTTTA
TGGGTGAAAAGAATAATTTGGGTCAAATGCAAGTGAAACCATATGGTGCATAATTTGAAA
GCCATATTATATCATTTGCTAAGTCCAAAGTAAAAATTTCACAAACTAGTTAGATTGCGA
TTTAGTCTATACACACTTCAACAGAGCTATATACACTAT

Figure 16.

NLFQIIMELQVIRIFRLVVACVLCLCIFIRSASSATKGFESIACCADSNYTDPKTTLTYT
TDHIWFSDKRSCRPIPEILFSHRSNKNVRIFEIDEGKRCYTLPTIKDQVYLIRGVFPFDS
LNSSFYVYIGVTELGELRSSRLEDLEIEGVFRATKDYIDFCLLKEDVNPFISQIELRPLP
EEYLHGFATSVLKLISRNNLGDINDDIRFPDDRNDRIWKRKATSTPSSALPLSFNVSNVD
LKDSVAPPLQVLQTALTHPERLEFVHDGLETDDYEYSVFLHFLELNGTVRAGQRVFDIYL
NNEIKKEKFDVLAGGSKNSYTALNISANGSLNITLVKASGSEFGPLLNAYEILQARSWIE
ETNQKDLELIQKTREELLLHNQENEALESWSGDPCMIFPWKGITCDDSTGSSIITMLDLS
SNNLKGAIPYFVTKMTNLQILNLSHNQFDSLFPSFPPSSLLISLDLSYNDLDGRLPESII
SLPHLKSLYFGCNPYMKDEDTTKLNSSLINTDYGRCKGKKPKFGQVFVIGAITSGSLLIT
LAVGILFFCRYRHKSITLEGFGGKTYPMATNIIFSLPSKDDFFIKSVSVKPFTLEYIEQA
TEQYKTLICEGGFGSVYRGTLDDGQEVAVKVRSSTSTQGTKEFDNELNLLSAIQHENLVP
LLGYCNEYDQQILVYPFMSNGSLLDRLYGEASKRKILDWPTRLSIALGAPRGLA*

Figure 17.

NLFQIMMELQVIRIFRLVVACVLCLCIFIRSASSATKGFESIACCADSNYTDPKTTLTYT
TDHIWFSDKRSCRPIPEILFSHRSNKNVRIFEIDEGKRCYTLPTIKDQVYLIRGVFPFDS
LNSSFYVYIGVTELGELRSSRLEDLEIEGVFRATKDYIDFCLLKEDVNPFISQIELRPLP
EEYLHGFATSVLKLISRNNLGDTNDDIRFPDDQNDRIWKRKATSTPSSALPLSSNVSNVD
LKDSVTPPLQVLQTALTHPERLEFVHDGLETDDYEYSVFLHFLELNGTVRAGQRVFDIYL
NNEIKKEKFDVLAGGSKNSYTALNISANGSLNITLVKASGSEFGPLLNAYEILQARSWIE
ETNQKDLELIQKMREELLLHNRENEALESWSGDPCMIFPWKGITCDDSTGSSIITMLDLS
SNNLKGAIPYFVTKMTNLQILNLSHNQFDSLFPSFPPSSLLISLDLSYNDLDGRLPESII
SLPHLKSLYFGCNPYMKDEDTTKLNSSLINTDYGRCKGKKPKFGQVFVIGAITRGSLLIT
LAVGILFFCRYRHKSITLEGFGGKTYPMATNIIFSLPSKDDFFIKSVSVKPFTLEYIEQA
TEQYKTLIGEGGFGSVYRGTLDDGQEVAVKVRSSTSTQGTXEFDNELNLLSAIQHENLVP
LLGYCNEYDQQILVYPFMSNGSLLDRLYGEASKRKILDWPTRLSIALGAARGLAYLHTFP
GRSVIHRDVKSSNILLDQSMCAKVADFGFSKYAPQEGDSYVSLEVRGTAGYLDPEYYKTQ
QLSEKSDVFSFGVVLLEIVSGREPLNIKRPRIEWSLVEWAKPYIRASKVDEIVDPGIKGG
YHAEALWRVVEVALQCLEPYSTYRPCMVDIVRELEDALIIENNASEYMKSIDSLGGSNRY
SIVMDKRALPSTTSTAESTITTQTLTHPQPR*

25/60

Figure 18. (1/6)

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.....TTCCAGGCCTAAAGTCAAACACCATATTTTAACAATATTCTTTCTTCTAC--------AGCTTTTATACAAGTT  MtA17
     TTCCAGGCCTAAAGTCAAACACCATATTTTAACAATATTCTTTCTTCTACAAGGGTATAACTTTTATACAAGTT  Ms1N-
                          CCATATTTTAACAATATTCTTTCTTCTACAAGGGTATAACTTTTATACAAGTT  Ms6N+
TTTTTTAACAATTTTTCTTTTTCTAATTTCTAACCCTCCATCAAATTGAGAACTTTTGTAAAATGGTTTCAAATCCTTT  Ps

CACTATATTATAGGATTGATCAGGGTTCATTTTTTCTTTCTTTGAAAAATCTCTAAGGGGTGCTGTTTCCAAGGCAGAG  MtA17
CACTATATTATAGGATTGATCAAGGTTCATTTTTTCTTTCTTTGAAAAATCTCTAAGGGGTGCTGTTTCCAAGGCAGAA  Ms1N-
CACTATATTATAGGATTGATCAAGGTTCATTTTTTCTTTCTTTGAAAAATCTCTAAGGGGTGTGGTTTCCAAGGCAGAA  Ms6N+
   CACAAAGGTATAACTTTTGTACATGTTTACTATATTAGAGGATTGATCAAGTTCCCTTTGAAAAATCTCTAGGGGG  Ps

AATGAAATAGAATTCAGAAGAATTTTTATGTTACTATAAAGGAAAGATGAAAAGTTAGTTAGCATGGATTCAAGTTTGA  MtA17
AATGAAATAGAATTCAGAAGAATTTTTATGGTACT--AAAGGGAAGATGAGAAGTTAGTTAGCATGGATTCAAATTTGA  Ms1N-
AATGAAATAGAATGCAGAAGAATTTGTATGGTACTATAAAGGGAAGATGAAAAGTTAGTTAGCATGGATTCAAGTTTGA  Ms6N+
  TGAAATAGAATTCAGAAGAATTTTTATGGTACTATA--GGGAAGATGGAGAGTTAGTTAGCATGGATTCGAGTTTGA  Ps

TAACCCTATGGGGTAAAATCTCTTTCAGATT ATG ATG GAG TTA CAA GTT ATT AGG ATA TTT AGA TTG  MtA17
TAACCCTTTGGGGTAAAATCTCTTTCAGATT ATA ATG GAG CTA CAA GTT ATT AGG ATA TTT AGA TTG  Ms1N-
TAACCCTTTGGGGTAAAATCTCTTTCAGATT ATG ATG GAG CTA CAA GTT ATT AGG ATA TTT AGA TTG  Ms6N+
GAACCCTTTGGGGTAACCTATCTTTCGGATT ATG ATG GAG CTA CGA GTT ATT TGT ATA ATA AGA TTG  Ps

GTT GTG GCA TTT GTT CTT TGT TTG TGT ATA TTT ATC AGA TCA GCT TCT TCT GCA ACT AAA  MtA17
GTT GTG GCA TGT GTT CTT TGT TTG TGT ATA TTT ATC AGA TCA GCT TCT TCT GCA ACT AAA  Ms1N-
GTT GTG GCA TGT GTT CTT TGT TTG TGT ATA TTT ATC AGA TCA GCT TCT TCT GCA ACT AAA  Ms6N+
GTT GTG GCG TGT GTT CTT TGT CTG TGT ATA TTT ATC AGA TCA GCT TCT TCT GCA ACT GAA  Ps

G i1 GG TTT GAG AGC ATA GCA TGT TGT GCT GAT TCA AAT TAC ACA GAT CCA AAA ACC ACC  MtA17
G i1 GG TTT GAG AGC ATA GCA TGT TGT GCT GAT TCC AAT TAC ACA GAT CCA AAA ACC ACC  Ms1N-
G i1 GG TTT GAG AGC ATA GCA TGT TGT GCT GAT TCC AAT TAC ACA GAT CCA AAA ACA ACC  Ms6N+
G i1 GG TTT GAG AGC ATA GCA TGC TGT GCT GAT TCG AA                               Ps

CTA ACT TAT ACA ACA GAT CAC ATC TGG TTC TCT GAT AAA AGA AGT TGC AGA CAA ATA CCC  MtA17
CTA ACT TAT ACA ACA GAT CAC ATC TGG TTC TCT GAT AAA AGA AGT TGC AGA CCA ATA CCC  Ms1N-
CTA ACT TAT ACA ACA GAT CAC ATC TGG TTC TCT GAT AAA AGA AGT TGC AGA CCA ATA CCC  Ms6N+

GAA ATT TTG TTT AGC CAC AGA AGC AAT AAA AAT GTT CGA AAA TTT GAA ATA TAT GAA GGA  MtA17
GAA ATT TTG TTT AGC CAC AGA AGC AAT AAA AAT GTT CGA ATA TTT GAA ATA GAT GAA GGA  Ms1N-
GAA ATT TTG TTT AGC CAC AGA AGC AAT AAA AAT GTT CGA ATA TTT GAA ATA GAT GAA GGA  Ms6N+

AAG AGA TGT TAT AAT TTG CCA ACA GTT AAG GAT CAA GTA TAT TTG ATA AGG GGC ATA TTT  MtA17
AAG AGA TGT TAT ACT TTG CCA ACA ATT AAG GAT CAA GTA TAT TTG ATA AGG GGT GTA TTT  Ms1N-
AAG AGA TGT TAT ACT TTG CCA ACA ATT AAG GAT CAA GTA TAT TTG ATA AGG GGT GTA TTT  Ms6N+

CCC TTT GAT AGT TTA AAT TCT TCG TTT TAT GTT TCG ATC GGG GTA ACA GAA CTA GGC GAA  MtA17
CCC TTT GAT AGT TTA AAT TCT TCG TTT TAT GTT TAT ATC GGG GTA ACA GAA CTA GGT GAA  Ms1N-
CCC TTT GAT AGT TTA AAT TCT TCG TTT TAT GTT TAT ATC GGG GTA ACA GAA CTA GGT GAA  Ms6N+

TTA AGA TCG TCT AGG CTC GAG GAC TTG GAA ATT GAG GGA GTT TTT AGA GCC ACC AAA GAC  MtA17
TTA AGA TCG TCT AGA CTC GAG GAC TTG GAA ATT GAG GGA GTT TTT AGA GCC ACC AAA GAC  Ms1N-
TTA AGA TCG TCT AGA CTC GAG GAC TTG GAA ATC GAG GGA GTT TTT AGA GCC ACC AAA GAC  Ms6N+
                                                                          T  Vv

TAC ATA GAT TTC TGC TTA TTG AAG GAG GAT GTC AAT CCC TTC ATT TCT CAG ATT GAA TTG  MtA17
TAT ATT GAT TTC TGC TTA TTG AAG GAG GAT GTC AAT CCC TTC ATT TCT CAG ATT GAA TTG  Ms1N-
TAT ATT GAT TTC TGC TTA TTG AAG GAA GAT GTC AAT CCC TTC ATT TCT CAG ATT GAA TTG  Ms6N+
GGC ATA GAC TTC TGC TTA TTG AAG GAG GAT GCC AAT CCC TTC ATT TCT CAG CTT GAA CTG  Vv
```

Figure 18. (cont. 2/6)

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AGG CCA TTA CCT GAA GAA TAC CTA CAT GGT TTC GGT ACT AGT GTT TTA AAA CTG ATA AGC  MtA17
AGG CCA TTA CCT GAA GAA TAC CTA CAT GGT TTC GCT ACT AGT GTT TTA AAA CTG ATA AGC  Ms1N-
AGG CCA TTA CCT GAA GAA TAC CTA CAT GGT TTC GCT ACT AGT GTT TTA AAA CTG ATA AGC  Ms6N+
AGG CCA TTA CCT GAA GAA TAC ATG CAT GAT TTC TCT ACC AGC GTT TTA AAA CTG ATA AAC  Vv

AGA AAC AAT CTT GGT GAC ACA AAT GAT GAT ATA AG i2 G TTC CCA GAT GAC CAA AAT GAT  MtA17
AGA AAC AAT CTT GGT GAC ATA AAT GAT GAT ATC AG i2 G TTC CCA GAT GAC CGA AAT GAT  Ms1N-
AGA AAT AAT CTT GGT GAC ACA AAT GAT GAT ATA AG i2 G TTC CCA GAT GAC CAA AAT GAT  Ms6N+
AGA AAT AAT CTT TGT GGC ATA GAA GAC GAC ATC AG i2 G TTC CCT GTT GAC CAA AAT GAT  Vv

AGA ATC TGG AAA CGG AAA GAA ACT TCA ACT CCA ACA TCT GCC CTT CCA CTG TCT TTC AAT  MtA17
AGA ATC TGG AAA CGG AAA GCA ACT TCA ACT CCA TCA TCT GCC CTT CCA CTG TCT TTC AAT  Ms1N-
AGA ATC TGG AAA CGG AAA GCA ACT TCA ACT CCA TCA TCT GCC CTT CCC CTG TCT TCC AAT  Ms6N+
AGA ATC TGG AAA --- --- GCA ACT TCA ACT CCA TCA TAT GCT GTT CCA CTG TCT TTC AAT  Vv

GTC AGC AAT GTT GAC CTC AAA GAC AGT GTC ACA CCT CCT CTA CAA GTC CTA CAA ACA GCT  MtA17
GTC AGC AAT GTT GAC CTC AAA GAC AGT GTC GCA CCT CCT CTA CAA GTC CTA CAA ACA GCT  Ms1N-
GTC AGC AAT GTT GAC CTC AAA GAC AGT GTC ACA CCT CCT CTA CAA GTC CTA CAA ACA GCT  Ms6N+
GTC AGT GAT GTT GAT CTC AAC GGC AAA GTG ACA CCT CCT CGA CAA GTC CTA CAA ACA GCT  Vv

CTT ACT CAC CCT GAG CGA TTG GAG TTC GTC CAT GAT GGC CTC GAG ACC GAT GAT TAT GAA  MtA17
CTT ACT CAC CCT GAG CGA TTG GAG TTT GTC CAT GAT GGC CTC GAG ACC GAT GAT TAT GAA  Ms1N-
CTT ACT CAC CCT GAG CGA TTG GAG TTC GTC CAT GAT GGC CTC GAG ACC GAT GAT TAT GAA  Ms6N+
CTT ACT CAC CCT GAT CGA TTG GTG TTC GTC CAC GAC GGC CTC GAG ACC GAT GAT TAT GAA  Vv

TAC TCT GTG TTT CTC CAC TTT CTT GAA CTA AAT GGC ACT GTC AGA GCA GGA CAA AGG GTG  MtA17
TAC TCT GTG TTT CTC CAC TTT CTT GAA CTA AAT GGC ACT GTC AGA GCA GGA CAA AGG GTG  Ms1N-
TAC TCT GTG TTT CTC CAC TTT CTT GAA CTA AAT GGC ACT GTC AGA GCA GGA CAA AGG GTG  Ms6N+
TAC TCT GTG TTA CTC TAC TTT CTT GAA CTA AAT AAC ACT GTC AAA GCA GGA CAA AGG GTG  Vv

TTT GAC ATC TAT CTA AAC AAT GAG ATT AAA AAG GAG AAA TTT GAT GTT TTG GCT GGA GGG  MtA17
TTT GAC ATC TAT CTA AAC AAT GAG ATT AAA AAG GAG AAG TTT GAT GTT TTG GCT GGA GGG  Ms1N-
TTT GAC ATC TAT CTA AAC AAT GAG ATT AAA AAG GAG AAG TTT GAT GTT TTG GCT GGA GGG  Ms6N+
TTT GAC ATC TAT CTA AAC AGT GAG ATT AAA AAG GAG AGT TTT GAT GTA TCG GAA GGA GGA  Vv
                                                    TTT GAT GTA TTG GAA GGA GGA  Ps

TCC AAG AAC AGT TAC ACT GCC TTG AAC ATT TCA GCA AAT GGA TCA CTC AAT ATA ACC TTA  MtA17
TCC AAG AAC AGT TAC ACT GCC TTG AAC ATT TCA GCA AAT GGA TCA CTC AAT ATA ACC TTA  Ms1N-
TCC AAG AAC AGT TAC ACT GCC TTG AAC ATT TCA GCA AAT GGA TCA CTC AAT ATA ACC TTA  Ms6N+
TCC AAG TAC AGT TAC ATC ACC TTG AAC ATT TCA GCA AAT GGA TCA CTC AAT ATA ACC TTA  Vv
TCC AAG TAC AGT TAC ACT GTC TTG AAC ATT TCA GCA AAT GGA TCA CTC AAT ATA ACC TTA  Ps

GTC AAG GCA TCT GGA TCT GAG TTT GGA CCC CTT TTG AAT GCC TAT GAA ATC CTG CAG GCA  MtA17
GTC AAG GCA TCT GGA TCT GAG TTT GGA CCC CTT TTG AAT GCC TAT GAA ATC CTG CAG GCA  Ms1N-
GTC AAG GCA TCT GGA TCT GAG TTT GGA CCC CTT TTG AAT GCC TAT GAA ATC CTG CAG GCA  Ms6N+
GCC AAG GCA TCT GGA TCT AAG TTT GGA CCC CTT TTG AAT GCT TAT GAA ATC CTG CAG GCG  Vv
GTC AAG GCA TCT GGA TCT AAG TTT GGA CCC CTT TTG AAT GCC TAT GAG ATC CTG CAG GCG  Ps

CGG TCG TGG ATT GAA GAG ACC AAC CAA AAA GAT T i3 TG GAA GTT ATT CAG AAG ATG AGA  MtA17
CGG TCG TGG ATT GAA GAG ACC AAC CAA AAA GAT T i3 TG GAA CTT ATT CAG AAG ACA AGA  Ms1N-
CGG TCG TGG ATT GAA GAG ACC AAC CAA AAA GAT T i3 TG GAA CTT ATT CAG AAG ATG AGA  Ms6N+
CGA CCA TGG ATC GAT GAG ACC GAC CAA ACA GAT G i3 TG GAA GTT ATT CAG AAG TTG AGA  Vv
CGA CCA TGG ATC GAT GAG ACC GAC CAA ACA GAT C i3 TG GAA GTT ATT CAG AAG ATG AGA  Ps
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27/60

Figure 18. (cont. 3/6)

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GAA GAA CTG CTG CTG CAC AAC CAA GAA AAT GAA GCA TTG GAG AGT TGG AGT GGA GAC CCT  MtA17
GAA GAA CTG CTG CTG CAC AAC CAA GAA AAT GAA GCA TTG GAG AGT TGG AGT GGA GAC CCT  Ms1N-
GAA GAA CTG CTG CTG CAC AAC CGA GAA AAT GAA GCA TTG GAG AGT TGG AGT GGA GAC CCT  Ms6N+
AAA GAA CTA CTG CTG CAA AAC CAA GAC AAT GAA GCA TTG GAG AGT TGG AGT GGA GAT CCT  Vv
AAA GAA CTG CTG CTG CAA AAC CAA GAC AAT GAA GCA TTG GAG AGT TGG AGT GGA GAT CCT  Ps

TGT ATG ATT TTC CCC TGG AAA GGA ATA ACA TGT GAT GAT TCA ACT GGT TCA TCT ATT ATC  MtA17
TGT ATG ATT TTC CCC TGG AAA GGA ATA ACA TGT GAT GAT TCA ACT GGT TCA TCT ATT ATC  Ms1N-
TGT ATG ATT TTC CCC TGG AAA GGA ATA ACA TGT GAT GAT TCA ACT GGT TCA TCT ATT ATC  Ms6N+
TGT ATG CTT TTT CCC TGG AAA GGA GTA GCA TGC GAT GGT TCA AAT GGT TCG TCT GTC ATC  Vv
TGT ATG CTT TTT CCA TGG AAA GGA GTA GCA TGT GAT GGT TCA AAT GGT TCG TCT GTC ATC  Ps

ACT AAG CT i4 G GAT CTT TCT TCC AAT AAT CTC AAG GGA GCA ATT CCT TCC ATT GTC ACT  MtA17
ACT ATG CT i4 G GAT CTT TCT TCC AAT AAT CTC AAG GGA GCA ATT CCT TAC TTT GTC ACT  Ms1N-
ACT ATG CT i4 G GAT CTT TCT TCC AAT AAT CTC AAG GGA GCA ATT CCT TAC TTT GTC ACT  Ms6N+
ACT AAG CT i4 G GAT CTT TCC TCC AGT AAT CTC AAA GGA ACA ATC CCT TCC AGT GTC ACT  Vv
ACT AAG CT i4 G GAT CTT TCC TCC AGT AAT CTC AAA GGA ACA ATT CCT TCC AGT GTC ACT  Ps

AAG ATG ACC AAT TTA CAA ATA CT i5 G AAC CTG AGC CAC AAC CAG TTC GAT ATG TTA TTC  MtA17
AAG ATG ACC AAT TTA CAA ATA CT i5 G AAC CTG AGC CAC AAC CAG TTC GAT TCG TTA TTC  Ms1N-
AAG ATG ACC AAT TTA CAA ATA CT i5 G AAC CTG AGC CAC AAC CAG TTC GAT TCG TTA TTC  Ms6N+
GAG ATG ACC AAA TTA CAA ATA CT i5 G AAC CTG AGC CAC AAC CAC TTC GAT GGC TAT ATC  Vv
GAG ATG ACC AAA TTA CAA ATA CT i5 G AAC CTG AGC CAC AAC CAT TTC GAT GGT TAT ATC  Ps

CCC TCG TTT CCA CCG TCC TCC TTG CTG ATA TCA TT i6 G GAT CTT AGC TAC AAT GAT CTT  MtA17
CCC TCG TTT CCA CCG TCC TCC TTG CTG ATA TCA TT i6 G GAT CTG AGC TAC AAT GAT CTT  Ms1N-
CCC TCG TTT CCA CCG NCC TCC TTG CTG ATA TCA TT i6 G GAT CTG AGC TAC AAT GAT CTT  Ms6N+
CCC TCG TTT CCA TCG TCT TCC TTG TTG ATA TCA GT i6 A GAT CTG AGC TAC AAT GAC CTA  Vv
CCC TCA TTT CCA CCG TCC TCC TTG TTG ATA TCA GT i6 A GAT CTG AGC TAC AAT GAC CTA  Ps

TCA GGA TGG CTT CCA GAA TCC ATT ATC TCA CTG CCA CAT TTA AAA TCA TT i7 A TAT TTT  MtA17
GAT GGA CGG CTT CCA GAA TCC ATT ATC TCA CTG CCA CAT TTA AAA TCA TT i7 A TAT TTT  Ms1N-
GAT GGA CGG CTT CCA GAA TCC ATT ATC TCA CTG CCA CAT TTA AAA TCA TT i7 A TAT TTT  Ms6N+
ACG GGA CAG CTT CCA GAA TCC ATT ATC TCA CTG CCA CAT TTA AAC TCA TT i7 G TAT TTT  Vv
ACG GGA CAG CTT CCA GAA TCC ATT ATC TCA CTG CCA CAT TTA AAC TCA TT i7 A TAT TTT  Ps

GGC TGC AAT CCA TCT ATG AGT GAC GAA GAT ACA ACA AAG TTG AAC AGT TCA CTA ATC AAT  MtA17
GGC TGC AAT CCA TAT ATG AAG GAC GAA GAT ACA ACA AAG TTG AAC AGT TCA CTA ATC AAT  Ms1N-
GGC TGC AAT CCA TAT ATG AAG GAC GAA GAT ACA ACA AAG TTG AAC AGT TCA CTA ATC AAT  Ms6N+
GGT TGC AAT CAA CAC ATG AGC GAC GAT GAT GAA GCC AAA TTG AAC AGT TCA CTA ATC AGT  Vv
GGC TGC AAT CAA CAC ATG AGA GAC GAT GAT GAA GCC AAA TTG AAC AGT TCA CTA ATC AAT  Ps

ACA GA i8 T TAT GGG AGA TGC AAA GCA AAA AAA CCA AAG TTT GGA CAA GTA TTC GTG ATT  MtA17
ACA GA i8 T TAT GGG AGA TGC AAA GGA AAA AAA CCA AAG TTT GGA CAA GTA TTC GTG ATT  Ms1N-
ACA GA i8 T TAT GGG AGA TGC AAA GGA AAA AAA CCA AAG TTT GGA CAA GTA TTC GTG ATT  Ms6N+
ACA GA i8 T TAT GGG AGA TGC AAG GCA AAA AGC CCC AAA TTT GGA CAA GTA TTC ATG ATT  Vv
ACA GA i8 T TAT GGG AGA TGT AAT GCA AAA AAA CCC AAA TTT GGA CAA GTA TTC ATG ATT  Ps

GGA GCT ATT ACA AGT GGA TCA CTT TTG ATT ACT TTG GCT GTT GGA ATT CTA TTT TTT TGC  MtA17
GGA GCT ATT ACA AGT GGA TCA CTT TTG ATT ACT TTG GCT GTT GGA ATT CTA TTT TTT TGC  Ms1N-
GGA GCT ATT ACA AGT GGA TCA CTT TTG ATT ACT TTG GCT GTT GGA ATT CTA TTT TTT TGC  Ms6N+
GGA GCA ATT ACA AGT GGA TCG ATT TTG ATT ACT TTG GCT GTT GGA ATT CTA TTT TTT TGC  Vv
GGA GCT ATT ACA AGT GGA TCA ATT TTG ATT ACT TTG GCT GTT GTA ATT CTA TTC TTT TGC  Ps

CGT TAT AGA CAC AAG TCA ATT ACT TTG GAA GGA TTT GG- --A AAG ACC TAC CCA ATG GCA  MtA17
CGT TAT AGA CAC AAG TCA ATT ACT TTG GAA GGA TTT GGT GGA AAG ACC TAC CCA ATG GCA  Ms1N-
CGT TAT AGA CAC AAG TCA ATT ACT TTG GAA GGA TTT GGT GGA AAG ACC TAC CCA ATG GCA  Ms6N+
CGT TAT AGA CAC AAG TCA ATT ACT TTG GAA GGA TTT GGT GGA AAG ACC TAC CCG ATG GCA  Vv
CGT TAT AGA CAC AAG TCA ATT ACT TTG GAA GGA TTT GGT GGA AAG ACC TAC CCA ATG GCA  Ps
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28/60

Figure 18. (cont. 4/6)

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ACA A i9 AT ATA ATT TTC TCT TTG CCA AGC AAA GAC GAT TTC TTC ATA AAG TCT GTA TCA  MtA17
ACA A i9 AT ATA ATC TTC TCT TTG CCA AGC AAA GAC GAT TTC TTC ATA AAG TCT GTA TCA  Ms1N-
ACA A i9 AT ATA ATC TTC TCT TTG CCA AGC AAA GAC GAT TTC TTC ATA AAG TCT GTA TCA  Ms6N+
ACA A i9 AT ATA ATT TTC TCC TTG CCA AGC AAA GAC GAT TTC TTC ATA AAG TCT GTA TCA  Vv
ACA A i9 AT ATA ATT TTT TCC TTG CCA AGC AAA GAC GAT TTC TTC ATA AAG TCT GTA TCA  Ps

GTT AAA CCG TTC ACT TTG GAG TAT ATA GAG CAG GCT ACA GAA CAG TAC AAA ACT TTA ATA  MtA17
GTT AAA CCA TTC ACT TTG GAG TAT ATA GAG CAG GCT ACA GAA CAG TAC AAA ACT TTG ATA  Ms1N-
GTT AAA CCA TTC ACT TTG GAG TAT ATA GAG CAG GCT ACA GAA CAG TAC AAA ACT TTG ATA  Ms6N+
GTT AAA CCG TTC TCT TTG GAG TAT ATA GAG TTG GCA ACA GAG AAG TAC AAA ACT TTG ATA  Vv
GTT AAG CCA TTC ACT TTG GAG TAT ATA GAG TTG GCA ACA GAG AAG TAC AAA ACT TTG ATA  Ps

GGT GAA GGA GGA TTT GGC TCT GTT TAC AGG GGC ACT CTA GAC GAT GGT CAA GAA GTG GCA  MtA17
TGT GAA GGA GGA TTT GGT TCT GTT TAC AGA GGC ACT CTA GAC GAT GGT CAA GAA GTG GCA  Ms1N-
GGT GAA GGA GGA TTT GGT TCT GTT TAC AGA GGC ACT CTA GAC GAT GGT CAA GAA GTG GCA  Ms6N+
GGT GAA GGA GGG TTT GGC TCT GTT TAC CGA GGC ACT CTA GAC GAT GGT CAA GAA GTG GCA  Vv
GGT GAA GGA GGA TTT GGC TCT GTT TAC CGG GGC ACT CTA GAC GAT GGA CAA GAA GTG GCA  Ps

GTG AAA GTG CGG TCA TCC ACA TCA ACT CAG GGA ACC CGA GAA TTT GAT AAT GAG i10 CTA  MtA17
GTG AAA GTG CGG TCA TCC ACA TCA ACT CAG GGA ACC AAG GAA TTT GAT AAC GAG i10 CTA  Ms1N-
GTG AAA GTG CGG TCA TCC ACA TCA ACT CAG GGA ACC CGA GAA TTT GAT AAT GAG i10 CTA  Ms6N+
GTG AAA GTC CGG TCA GCC ACA TCA ACT CAG GGA ACC CGA GAA TTT GAC AAT GAG i10 CTA  Vv
GTG AAA GTC CGG TCA GCC ACA TCA ACT CAG GGA ACC CGA GAA TTT GAC AAT GAG i10 CTA  Ps

AAC CTA CTT TCA GCT ATA CAA CAT GAG AAC CTG GTG CCT CTT CTG GGT TAC TGT AAT GAG  MtA17
AAC CTA CTT TCA GCT ATA CAA CAT GAG AAC CTG GTG CCT CTT CTG GGT TAC TGT AAT GAG  Ms1N-
AAC CTA CTT TCA GCT ATA CAA CAT GAG AAC CTG GTG CCT CTT CTG GGT TAC TGT AAT GAG  Ms6N+
AAC CTA CTT TCA GCT ATA CAA CAT GAG AAC CTG GTG CCT CTT CTT GGT TAC TGT AAT GAA  Vv
AAC CTA CTT TCA GCT ATA CAA CAT GAG AAC CTG GTG CCT CTT CTT GGT TAC TGT AAT GAA  Ps

TAT GAT CAA CAA ATT CTC GTG TAT CCT TTC ATG TCC AAT GGC TCT TTG CTA GAT AGA CTA  MtA17
TAT GAT CAA CAA ATT CTC GTG TAT CCA TTC ATG TCT AAT GGC TCT TTG CTA GAT AGA TTA  Ms1N-
TAT GAT CAA CAA ATT CTC GTG TAT CCA TTC ATG TCC AAT GGC TCT TTG CTA GAT AGA CTA  Ms6N+
TAT GAT CAA CAA ATT CTT GTG TAC CCT TTC ATG TCT AAT GGA TCT TTG CTA GAT AGA CTA  Vv
TAT GAT CAA CAA ATT CTT GTA TAT CCT TTC ATG TCT AAT GGC TCT TTG CTA TAC AGA CTA  Ps

TAC G i11 GG GAA GCA TCA AAG AGA AAA ATA TTA GAC TGG CCA ACT AGA CTC TCT ATT GCT  MtA17
TAC G i11 GG GAA GCA TCA AAG AGA AAA ATA TTA GAC TGG CCA ACT AGA CTC TCT ATT GCT  Ms1N-
TAC G i11 GG GAA GCA TCA AAG AGA AAA ATA TTA GAC TGG CCA ACT AGA CTC TCT ATT GCT  Ms6N+
TAT G i11 GG GAA CCA GCA AAG AGA AAA ATA CTA GAC TGG CCA ACT AGA CTC TCT ATT GCT  Vv
TAT G i11 GG GAA CCG GCA AAG AGA AAA ATA CTA GAC TGG CCA ACT AGA CTC TCA ATT GCT  Ps

CTC GGT GCA GCT CGA G i12 GT TTG GCA TAT CTT CAC ACA TTT CCA GGA CGT TCT GTA ATA  MtA17
CTC GGT GCA CCT CGA G i12 GT TTG GCA TAG CTT CAC ACA TTT CCA GGA CCT TCT GTA ATA  Ms1N-
CTC GGT GCA GCT CGA G i12 GT TTG GCA TAA CTT CAC ACA TTT CCA GGA CGT TCT GTA ATA  Ms6N+
CTA GGA GCA GCT CGA G i12 GT TTG GCA TAT CTT CAC ACA TTT CCT GGA CGG TCT GTA ATA  Vv
CTA GGA GCA GCT CGA G i12 GT TTG GCA TAT CTT CAC ACG TTT CCT GGA CGA TCT GTA ATA  Ps
                          mutation resulting in in frame STOP codon

CAC AGG GAC GTA AAA TCG AGC AAT ATA CTG CTG GAT CAG AGC ATG TGT GCT AAG GTT GCA  MtA17
CAC AGG GAC CTA AAA TCG AGC AAT ATA CTG CTG GAT CAG AGC ATG TGT GCT AAG GTT GCA  Ms1N-
CAC AGG GAC GTA AAA TCG AGC AAT ATA CTG CTG GAT CAG AGC ATG TGT GCT AAG GTT GCA  Ms6N+
CAC AGG GAC GTA AAA TCG AGC AAT ATA CTA CTG GAC CAT AGC ATG TGT GCT AAG GTC GCA  Vv
CAC AGG GAC GTA AAA TCG AGC AAT ATA CTA CTG GAC CAT AGC ATG TGT GCT AAG GTT GCA  Ps
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29/60

Figure 18. (cont. 5/6)

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GAT TTT GGT TTC TCA AAA TAT GCT CCT CAG GAA GGA GAC AGT TAT GTT TCC CTT GAA GTA  MtA17
GAT TTT GGT TTC TCA AAA TAC GCT CCT CAG GAA GGA GAC AGT TAT GTT TCC CTT GAA GTA  Ms1N-
GAT TTT GGT TTC TCA AAA TAC GCT CCT CAG GAA GGA GAC AGT TAT GTT TCC CTT GAA GTA  Ms6N+
GAT TTT GGT TTC TCA AAA TAC GCT CCT CAG GAA GGA GAC AGT TAT GTT TCC CTT GAA GTA  Vv
GAT TTT GGT TTC TCA AAA TAC GCT CCT CAG GAA GGA GAC AGT TAT GTT TCC CTT GAA GTA  Ps

AGA GGA ACT GCA GGG TAT CTG GAT CCT GAG TAC TAC AAA ACC CAG CAA TTA TCT GAA AAA  MtA17
AGA GGA ACT GCA GGG TAT CTG GAT CCT GAG TAC TAC AAA ACC CAG CAA TTA TCT GAA AAA  Ms1N-
AGA GGA ACT GCA GGG TAT CTG GAT CCT GAG TAC TAC AAA ACC CAG CAA TTA TCT GAA AAA  Ms6N+
AGA GGA ACT GCA GGG TAT CTG GAT CCC GAG TAC TAC AAA ACC CAG CAA TTA TCT GAA AAG  Vv
AGA GGA ACC GCA GGA TAT CTG GAT CCT GAG TAC TAC AAA ACC CAG CAA TTA TCT GAA AAG  Ps

AGT GAT i13 GTT TTC AGC TTT GGT GTG GTT CTT CTT GAA ATT GTA AGC GGA CGG GAA CCT  MtA17
AGT GAT i13 GTT TTC AGC TTT GGT GTG GTT CTA CTT GAA ATT GTA AGT GGA CGG GAA CCT  Ms1N-
AGT GAT i13 GTT TTC AGC TTT GGT GTG GTT CTA CTT GAA ATT GTA AGT GGA CGG GAA CCT  Ms6N+
AGT GAT i13 GTT TTT AGT TTT GGC GTG GTT CTA CTA GAA ATT GTA AGT GGC CGG GAA CCT  Vv
AGT GAT i13 GTT TTC AGC TTT GGC GTG GTT CTA CTA GAA ATT GTA AGT GGC CGG GAA CCT  Ps

CTC AAC ATA AAG AGA CCA AGG ATC GAG TGG AGC TTG GTT GAA TGG i14 GCT AAA CCA TAC  MtA17
CTC AAC ATA AAG AGA CCA CGG ATC GAG TGG AGC TTG GTT GAA TGG i14 GCT AAA CCA TAC  Ms1N-
CTC AAC ATA AAG AGA CCA CGG ATC GAG TGG AGC TTG GTT GAA TGG i14 GCT AAA CCA TAC  Ms6N+
CTC AAC ATA AAG AGA CCA CGA GTC GAG TGG AGC TTG GTT GAA TGG i14 GCT AAA CCA TAC  Vv
CTC AAC ATA AAG AGA CCA CGA GTC GAG TGG AGC TTG GTT GAA TGG i14 GCT AAA CCA TAT  Ps

ATA AGA GCA TCA AAG GTG GAT GAA ATT GTA GAT CCT GGC ATC AAG GGA GGA TAT CAT GCA  MtA17
ATA AGA GCA TCA AAG GTG GAT GAA ATT GTA GAT CCT GGC ATC AAG GGA GGA TAT CAT GCA  Ms1N-
ATA AGA GCA TCA AAG GTG GAT GAA ATT GTA GAT CCT GGC ATC AAG GGA GGA TAT CAT GCA  Ms6N+
ATA AGA GCA TCA AAG GTG GAT GAA ATT GTA GAT CCT GGC ATC AAG GGA GGA TAT CAT GCA  Vv
ATA AGA GCA TCA AAG GTG GAT GAA ATT GTA GAT CCT GGC ATC AAG GGA GGA TAT CAT GCA  Ps


GAG GCA TTG TGG AGA GTT GTG GAA GTA GCA CTA CAA TGT CTA GAA CCC TAC TCA ACA TAT  MtA17
GAA GCA TTG TGG AGA GTT GTG GAA GTA GCA CTG CAA TGT CTA GAA CCC TAC TCA ACA TAT  Ms1N-
GAA GCA TTG TGG AGA GTT GTG GAA GTA GCA CTG CAA TGT CTA GAA CCC TAC TCA ACA TAT  Ms6N+
GAG GCA CTG TGG AGA GTG GTG GAA GTA GCA CTG CAA TGT CT                           Vv
GAG GCA CTG TGG AGA GTT                                                         Ps

CGG CCA TGC ATG GTT GAT ATT GTC CGC GAG TTG GAG GAT GCT CTC ATT ATT GAA AAC AAT  MtA17
AGG CCA TGC ATG GTT GAT ATT GTC CGC GAG TTG GAG GAT GCT CTC ATT ATT GAA AAC AAT  Ms1N-
AGG CCA TGC ATG GTT GAT ATT GTC CGC GAG TTG GAG GAT GCT CTC ATT ATT GAA AAC AAT  Ms6N+


GCA TCT GAA TAC ATG AAA TCC ATA GAC AGC CTT GGA GGA TCC AAC CGC TAC TCA ATT GTT  MtA17
GCA TCT GAA TAC ATG AAA TCC ATA GAC AGC CTT GGA GGA TCC AAC CGC TAC TCA ATT GTT  Ms1N-
GCA TCT GAA TAC ATG AAA TCC ATA GAC AGC CTT GGA GGA TCC AAC CGC TAC TCA ATT GTT  Ms6N+

ATG GAC AAA CGG GCG CTG CCT TCA ACT ACA TCT ACA GCA GAA TCA ACT ATC ACA ACC CAA  MtA17
ATG GAC AAA CGG GCG CTG CCT TCA ACT ACA TCT ACA GCA GAA TCA ACT ATC ACA ACC CAA  Ms1N-
ATG GAC AAA CGG GCG CTG CCT TCA ACT ACA TCT ACA GCA GAA TCA ACT ATC ACA ACC CAA  Ms6N+

ACC TTG TCA CAC CCT CAA CCG AGA TAG TAA ATGGGTCGA--TGGAATTCTTTTGATTTGTTTTTGATCATT  MtA17
ACC TTG ACA CAC CCT CAA CCG AGA TAG TAA ATGGGTCGA--TGGAATTCTTTTGATTTGTTTTTGATCATT  Ms1N-
ACC TTG ACA CAC CCT CAA CCG AGA TAG TAA ATGGGTCGA--TGGAATTCTTTTGATTTGTTTTTTATCATT  Ms6N+
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30/60

Figure 18. (cont. 6/6)

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GCTTTAGTAATATCACATTTTAAATGGTAAAGGAGAAAAATACTACTTCTGATTGTATTTCCATCCACTCTATGTTTCT  MtA17
GCTTTAGTAATATCCCATTTTCAATGGTAAAGGAGAAAAATACTACTTTTGATTGTATTTTCATCCACTCTATGTTTCT  Ms1N-
GCTTTAGTAATATCCCATTTTAAATGGTAAAGGAGAAAAATACTACTTTTGATTGTATTTTCATCCACTCTATGTTTCT  Ms6N+

TGAAACTGAATCTCTCTTGCTCAGCCCCAGTTTTTATGGGTGAAAAGAATAATTTGGGTCAAATGCAAGTGAAACCATA  MtA17
TGAAACTGAATCTCTCTTGCTCAGCCCCAGTTTTTATGGGTGAAAAGAATAATTTGGGTCAAATGCAAGTGAAACCATA  Ms1N-
TGAAACTGAATCTCTCTTGCTCAGCCCCAGTTTTTATGGGTGAAAAGAATAATTTGGGTCAAATGCAAGTGAAACCATA  Ms6N+

TGGTGCATAATTTAAAAGCCATATCATATCATTTGCCAAGTCCAAAGTAAAAATTTCACAAACTAGTTAGATTGCGATT  MtA17
TGGTGCATAATTTGAAAGCCATATTATATCATTTGCCAAGTCCAAAGTAAAAATTTCACAAACTAGTTAGATTGCGATT  Ms1N-
TGGTGCATAATTTGAAAGCCATATTATATCATTTGCTAAGTCCAAAGTAAAAATTTCACAAACTAGTTAGATTGCGATT  Ms6N+

TAGTCTATAGACACTTCAACAGAGCTATATACACTATGGTTGACTTGCGA                               MtA17
TAGTCTATAGACACTTCAACAGAGCTATATACACTATGGTTGACTTGCGACTAATTC                        Ms1N-
TAGTCTATACACACTTCAACAGAGCTATATACACTAT                                            Ms6N+
```

Figure 19. (1/2)

```
MMELQVIRIFRLVVAFVLCLCIFIRSASSATKGFESIACCADSNYTDPKTTLTYTTDHIW MtA17
 IMELQVIRIFRLVVACVLCLCIFIRSASSATKGFESIACCADSNYTDPKTTLTYTTDHIW Ms1N-
MMELQVIRIFRLVVACVLCLCIFIRSASSATKGFESIACCADSNYTDPKTTLTYTTDHIW Ms6N+
 MELRVICIIRLVVACVLCLCIFIRSASSATEGFESIACCADS                   Ps

FSDKRSCRQIPEILFSHRSNKNVRKFEIYEGKRCYNLPTVKDQVYLIRGIFPFDSLNSSF MtA17
FSDKRSCRPIPEILFSHRSNKNVRIFEIDEGKRCYTLPTIKDQVYLIRGVFPFDSLNSSF Ms1N-
FSDKRSCRPIPEILFSHRSNKNVRIFEIDEGKRCYTLPTIKDQVYLIRGVFPFDSLNSSF Ms6N+

YVSIGVTELGELRSSRLEDLEIEGVFRATKDYIDFCLLKEDVNPFISQIELRPLPEEYLH MtA17
YVYIGVTELGELRSSRLEDLEIEGVFRATKDYIDFCLLKEDVNPFISQIELRPLPEEYLH Ms1N-
YVYIGVTELGELRSSRLEDLEIEGVFRATKDYIDFCLLKEDVNPFISQIELRPLPEEYLH Ms6N+
                              GIDFCLLKEDANPFISQLELRPLPEEYMH Vv

GFGTSVLKLISRNNLGDTNDDIRFPDDQNDRIWKRKETSTPTSALPLSFNVSNVDLKDSV MtA17
GFATSVLKLISRNNLGDINDDIRFPDDRNDRIWKRKATSTPSSALPLSFNVSNVDLKDSV Ms1N-
GFATSVLKLISRNNLGDTNDDIRFPDDQNDRIWKRKATSTPSSALPLSSNVSNVDLKDSV Ms6N+
DFSTSVLKLINRNNLCGIEDDIRFPVDQNDRIWK--ATSTPSYAVPLSFNVSDVDLNGKV Vv

TPPLQVLQTALTHPERLEFVHDGLETDDYEYSVFLHFLELNGTVRAGQRVFDIYLNNEIK MtA17
APPLQVLQTALTHPERLEFVHDGLETDDYEYSVFLHFLELNGTVRAGQRVFDIYLNNEIK Ms1N-
TPPLQVLQTALTHPERLEFVHDGLETDDYEYSVFLHFLELNGTVRAGQRVFDIYLNNEIK Ms6N+
TPPRQVLQTALTHPDRLVFVHDGLETDDYEYSVLLYFLELNNTVKAGQRVFDIYLNSEIK Vv

KEKFDVLAGGSKNSYTALNISANGSLNITLVKASGSEFGPLLNAYEILQARSWIEETNQK MtA17
KEKFDVLAGGSKNSYTALNISANGSLNITLVKASGSEFGPLLNAYEILQARSWIEETNQK Ms1N-
KEKFDVLAGGSKNSYTALNISANGSLNITLVKASGSEFGPLLNAYEILQARSWIEETNQK Ms6N+
KESFDVSEGGSKYSYITLNISANGSLNITLAKASGSKFGPLLNAYEILQARPWIDETDQT Vv
   FDVLEGGSKYSYTVLNISANGSLNITLVKASGSKFGPLLNAYEILQARPWIDETDQT Ps

DLEVIQKMREELLLHNQENEALESWSGDPCMIFPWKGITCDDSTGSSIITKLDLSSNNLK MtA17
DLELIQKTREELLLHNQENEALESWSGDPCMIFPWKGITCDDSTGSSIITMLDLSSNNLK Ms1N-
DLELIQKMREELLLHNRENEALESWSGDPCMIFPWKGITCDDSTGSSIITMLDLSSNNLK Ms6N+
DVEVIQKLRKELLLQNQDNEALESWSGDPCMLFPWKGVACDGSNGSSVITKLDLSSSNLK Vv
DLEVIQKMRKELLLQNQDNEALESWSGDPCMLFPWKGVACDGSNGSSVITKLDLSSSNLK Ps

GAIPSIVTKMTNLQILNLSHNQFDMLFPSFPPSSLLISLDLSYNDLSGWLPESIISLPHL MtA17
GAIPYFVTKMTNLQILNLSHNQFDSLFPSFPPSSLLISLDLSYNDLDGRLPESIISLPHL Ms1N-
GAIPYFVTKMTNLQILNLSHNQFDSLFPSFPPSSLLISLDLSYNDLDGRLPESIISLPHL Ms6N+
GTIPSSVTEMTKLQILNLSHNHFDGYIPSFPSSSLLISVDLSYNDLTGQLPESIISLPHL Vv
GTIPSSVTEMTKLQILNLSHNHFDGYIPSFPPSSLLISVDLSYNDLTGQLPESIISLPHL Ps
```

Figure 19. (2/2)

```
KSLYFGCNPSMSDEDTTKLNSSLINTDYGRCKAKKPKFGQVFVIGAITSGSLLITLAVGI MtA17
KSLYFGCNPYMKDEDTTKLNSSLINTDYGRCKGKKPKFGQVFVIGAITSGSLLITLAVGI Ms1N-
KSLYFGCNPYMKDEDTTKLNSSLINTDYGRCKGKKPKFGQVFVIGAITSGSLLITLAVGI Ms6N+
NSLYFGCNQHMSDDDEAKLNSSLISTDYGRCKAKSPKFGQVFMIGAITSGSILITLAVGI Vv
NSLYFGCNQHMRDDDEAKLNSSLINTDYGRCNAKKPKFGQVFMIGAITSGSILITLAVVI Ps

LFFCRYRHKSITLEGFG-KTYPMATNIIFSLPSKDDFFIKSVSVKPFTLEYIEQATEQYK MtA17
LFFCRYRHKSITLEGFGGKTYPMATNIIFSLPSKDDFFIKSVSVKPFTLEYIEQATEQYK Ms1N-
LFFCRYRHKSITLEGFGGKTYPMATNIIFSLPSKDDFFIKSVSVKPFTLEYIEQATEQYK Ms6N+
LFFCRYRHKSITLEGFGGKTYPMATNIIFSLPSKDDFFIKSVSVKPFSLEYIELATEKYK Vv
LFFCRYRHKSITLEGFGGKTYPMATNIIFSLPSKDDFFIKSVSVKPFTLEYIELATEKYK Ps

TLIGEGGFGSVYRGTLDDGQEVAVKVRSSTSTQGTREFDNELNLLSAIQHENLVPLLGYC MtA17
TLICEGGFGSVYRGTLDDGQEVAVKVRSSTSTQGTKEFDNELNLLSAIQHENLVPLLGYC Ms1N-
TLIGEGGFGSVYRGTLDDGQEVAVKVRSSTSTQGTXEFDNELNLLSAIQHENLVPLLGYC Ms6N+
TLIGEGGFGSVYRGTLDDGQEVAVKVRSATSTQGTREFDNELNLLSAIQHENLVPLLGYC Vv
TLIGEGGFGSVYRGTLDDGQEVAVKVRSATSTQGTREFDNELNLLSAIQHENLVPLLGYC Ps

NEYDQQILVYPFMSNGSLLDRLYGEASKRKILDWPTRLSIALGAARGLAYLHTFPGRSVI MtA17
NEYDQQILVYPFMSNGSLLDRLYGEASKRKILDWPTRLSIALGAPRGLA*            Ms1N-
NEYDQQILVYPFMSNGSLLDRLYGEASKRKILDWPTRLSIALGAARGLAYLHTFPGRSVI Ms6N+
NEYDQQILVYPFMSNGSLLDRLYGEPAKRKILDWPTRLSIALGAARGLAYLHTFPGRSVI Vv
NEYDQQILVYPFMSNGSLLDRLYGEPAKRKILDWPTRLSIALGAARGLAYLHTFPGRSVI Ps

HRDVKSSNILLDQSMCAKVADFGFSKYAPQEGDSYVSLEVRGTAGYLDPEYYKTQQLSEK MtA17
HRDVKSSNILLDQSMCAKVADFGFSKYAPQEGDSYVSLEVRGTAGYLDPEYYKTQQLSEK Ms6N+
HRDVKSSNILLDHSMCAKVADFGFSKYAPQEGDSYVSLEVRGTAGYLDPEYYKTQQLSEK Vv
HRDVKSSNILLDHSMCAKVADFGFSKYAPQEGDSYVSLEVRGTAGYLDPEYYKTQQLSEK Ps

SDVFSFGVVLLEIVSGREPLNIKRPRIEWSLVEWAKPYIRASKVDEIVDPGIKGGYHAEA MtA17
SDVFSFGVVLLEIVSGREPLNIKRPRIEWSLVEWAKPYIRASKVDEIVDPGIKGGYHAEA Ms6N+
SDVFSFGVVLLEIVSGREPLNIKRPRVEWSLVEWAKPYIRASKVDEIVDPGIKGGYHAEA Vv
SDVFSFGVVLLEIVSGREPLNIKRPRVEWSLVEWAKPYIRASKVDEIVDPGIKGGYHAEA Ps

LWRVVEVALQCLEPYSTYRPCMVDIVRELEDALIIENNASEYMKSIDSLGGSNRYSIVMD MtA17
LWRVVEVALQCLEPYSTYRPCMVDIVRELEDALIIENNASEYMKSIDSLGGSNRYSIVMD Ms6N+
LWRVVEVALQCL                                                  Vv
LWRV                                                          Ps

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KRALPSTTSTAESTITTQTLTHPQPR**                                  Ms6N+
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Figure 20.

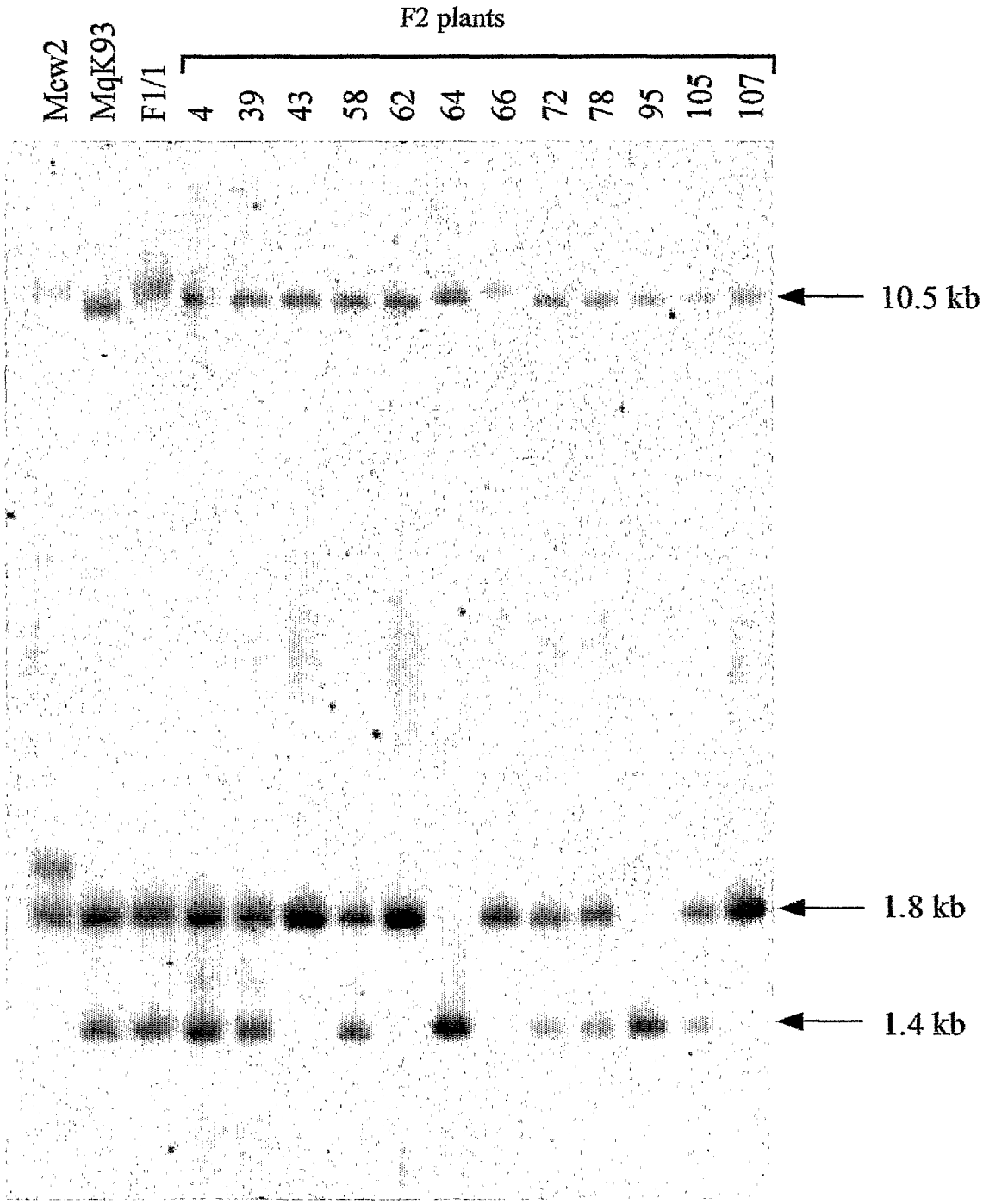
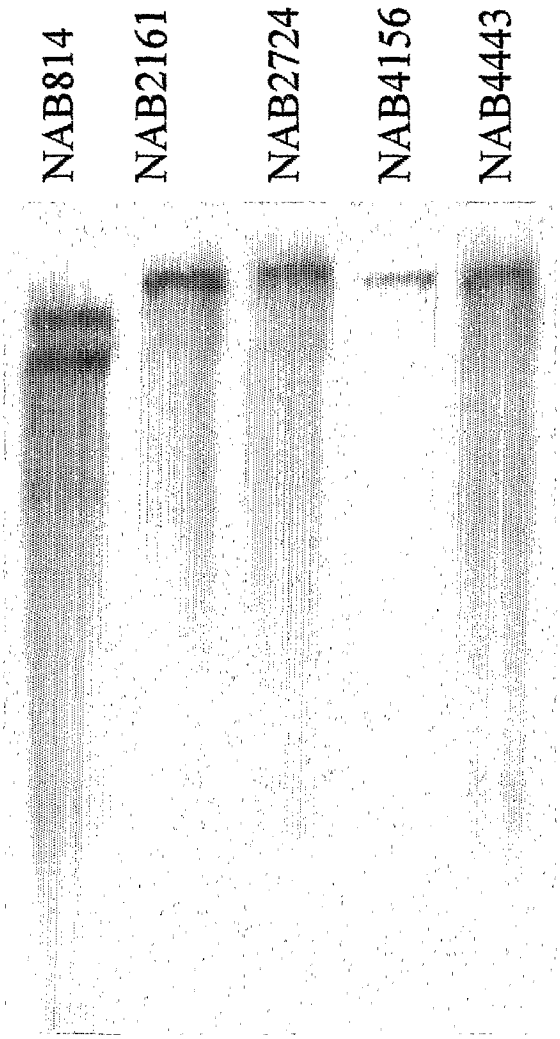

F2 plants
Mcw2
MqK93
F1/1
4
39
43
58
62
64
66
72
78
95
105
107
10.5 kb
1.8 kb
1.4 kb

Figure 21.



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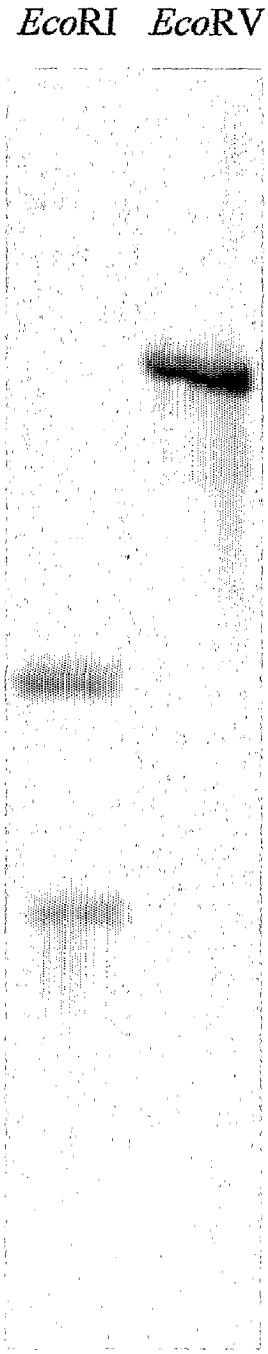

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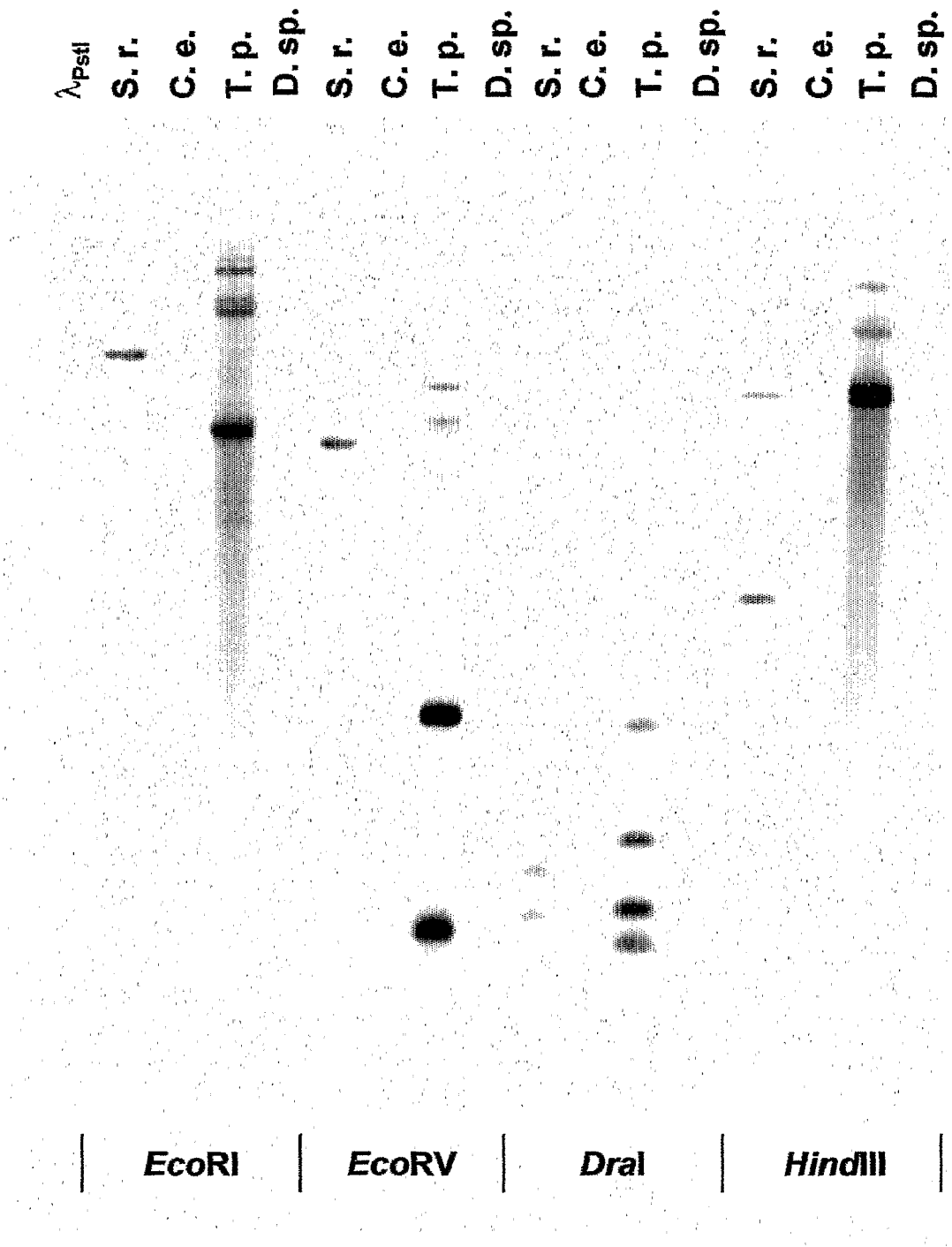
λPstI
S. r.
C. e.
T. p.
D. sp.
S. r.
C. e.
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D. sp.
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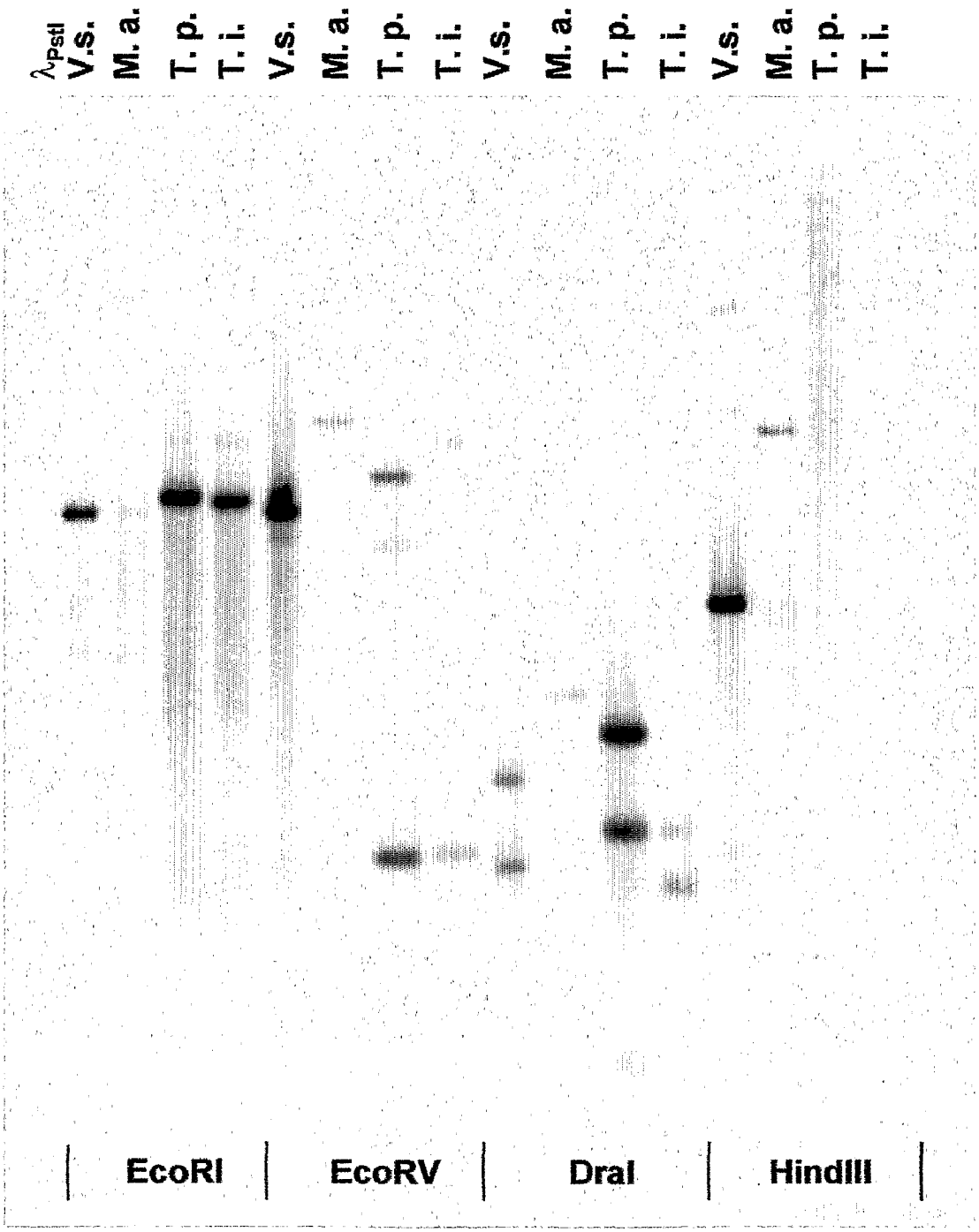
C. e.
T. p.
D. sp.
S. r.
C. e.
T. p.
D. sp.
EcoRI
EcoRV
DraI
HindIII

37/60

Figure 24.



38/60

Figure 25.

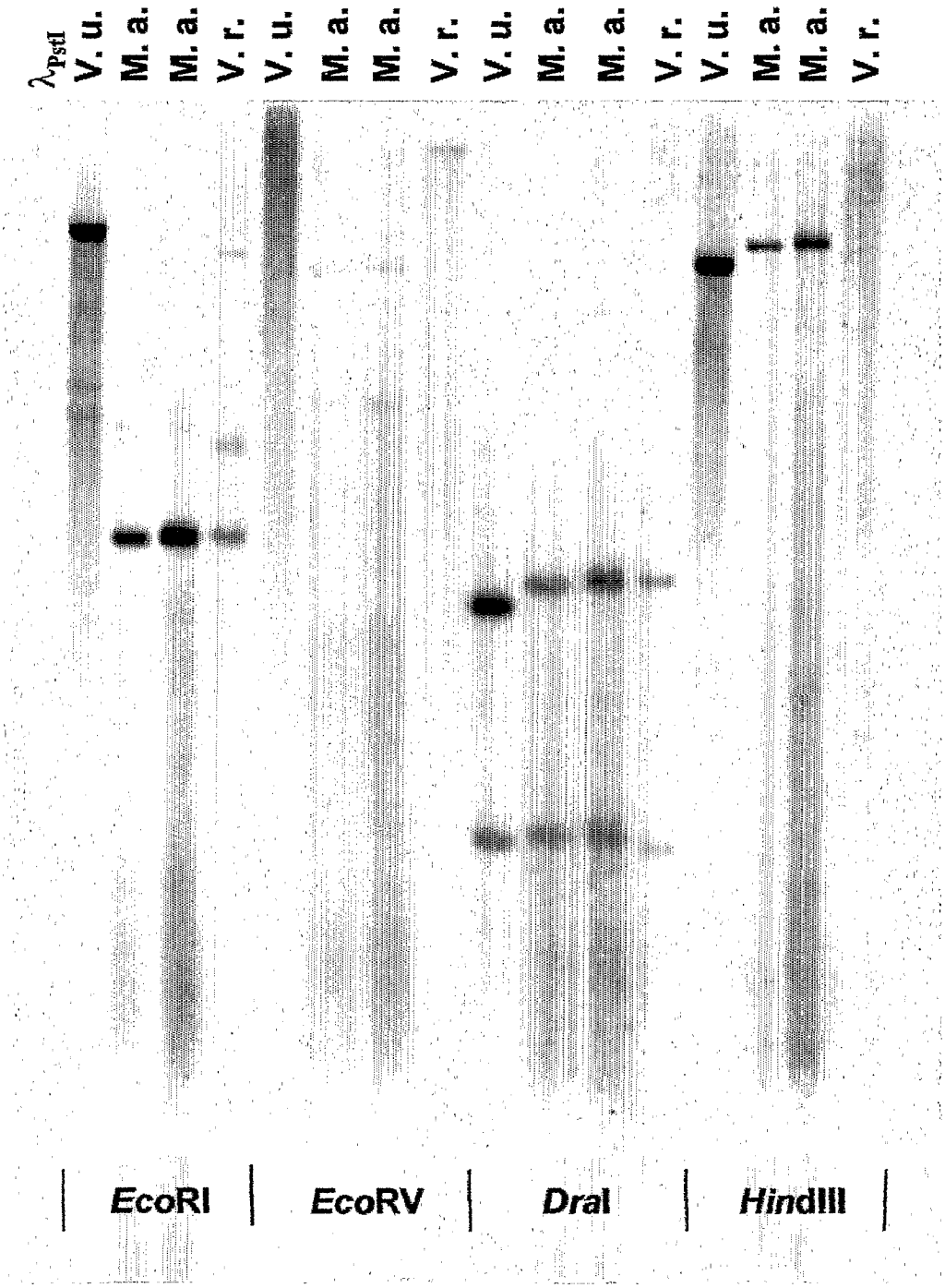
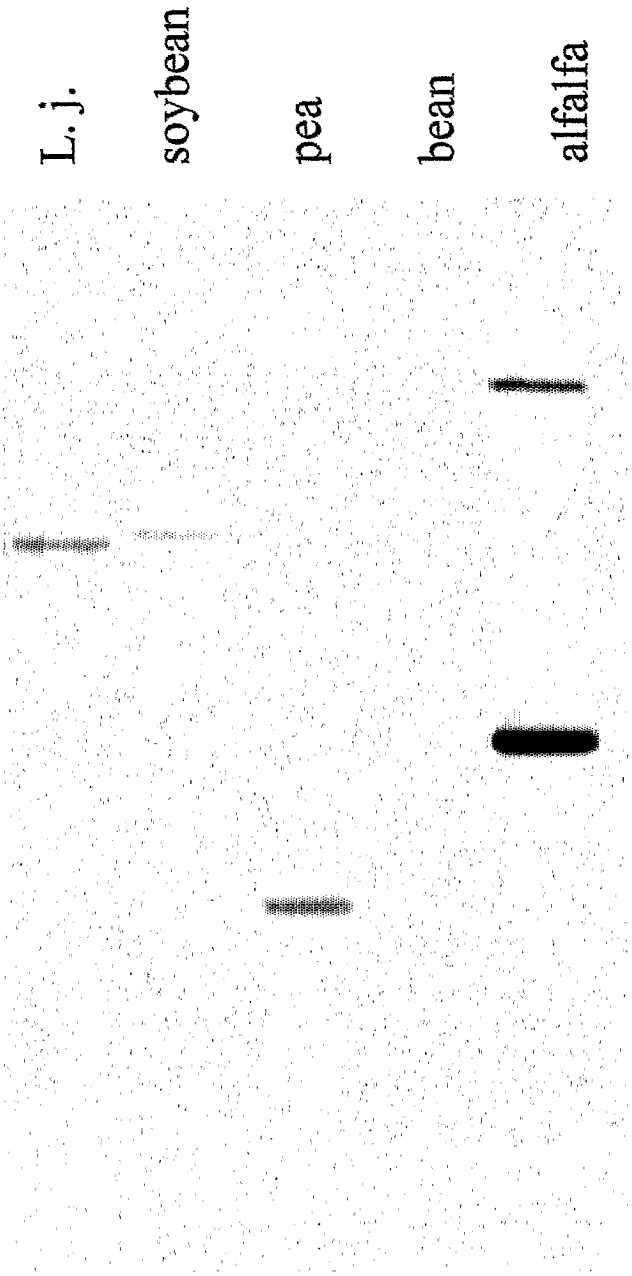


Figure 26.



L. j. = *Lotus japonicus* cv. funakura

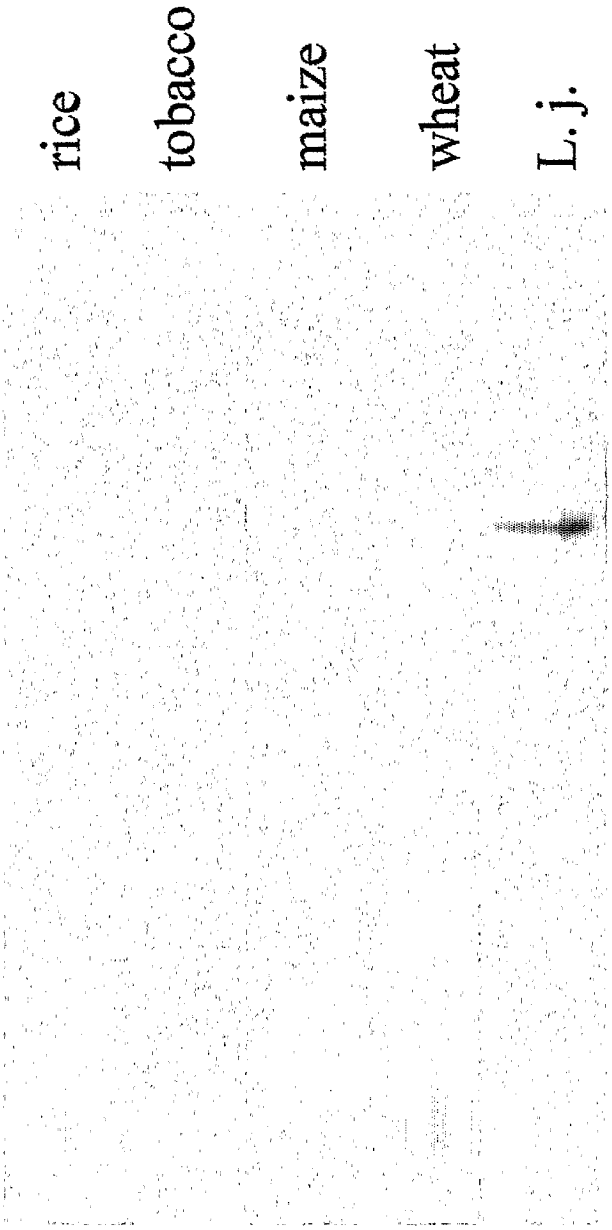
soybean = *Glycine max* cv. Williams

pea = *Pisum sativum* cv. újmajori

bean = *Phaseolus vulgaris* cv. Juliska

alfalfa = *Medicago sativa* ssp. *sativa* (*Eco*RI digestion)

Figure 27.



rice = Oryza sativa

tobacco = *Nicotiana tabacum* cv. Small Havanna SR1

maize = *Zea mays*

wheat = *Triticum aestivum*

L. j. = *Lotus japonicus* cv. funakura

Figure 28.

```
TTTTTTAACAATTTTTCTTTTTCTAATTTCTAACCCTCCATCAAATTGAGAACTTTTGTA
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GGGTAACCTATCTTTCGGATTATGATGGAGCTACGAGTTATTTGTATAATAAGATTGGTT
GTGGCGTGTGTTCTTTGTCTGTGTATATTTATCAGATCAGCTTCTTCTGCAACTGAAGGG
TTTGAGAGCATAGCATGCTGTGCTGATTCGAANNNNNNNNNNNNNNNNNNNNNNNNNNNN
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GTCTTGAACATTTCAGCAAATGGATCACTCAATATAACCTTAGTCAAGGCATCTGGATCT
AAGTTTGGACCCCTTTTGAATGCCTATGAGATCCTGCAGGCGCGACCATGGATCGATGAG
ACCGACCAAACAGATCTGGAAGTTATTCAGAAGATGAGAAAAGAACTGCTGCTGCAAAAC
CAAGACAATGAAGCATTGGAGAGTTGGAGTGGAGATCCTTGTATGCTTTTTCCATGGAAA
GGAGTAGCATGTGATGGTTCAAATGGTTCGTCTGTCATCACTAAGCTGGATCTTTCCTCC
AGTAATCTCAAAGGAACAATTCCTTCCAGTGTCACTGAGATGACCAAATTACAAATACTG
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GCCAAATTGAACAGTTCACTAATCAATACAGATTATGGGAGATGTAATGCAAAAAAACCC
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CTTGGTTACTGTAATGAATATGATCAACAAATTCTTGTATATCCTTTCATGTCTAATGGC
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CGATCTGTAATACACAGGGACGTAAAATCGAGCAATATACTACTGGACCATAGCATGTGT
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42/60

Figure 29.

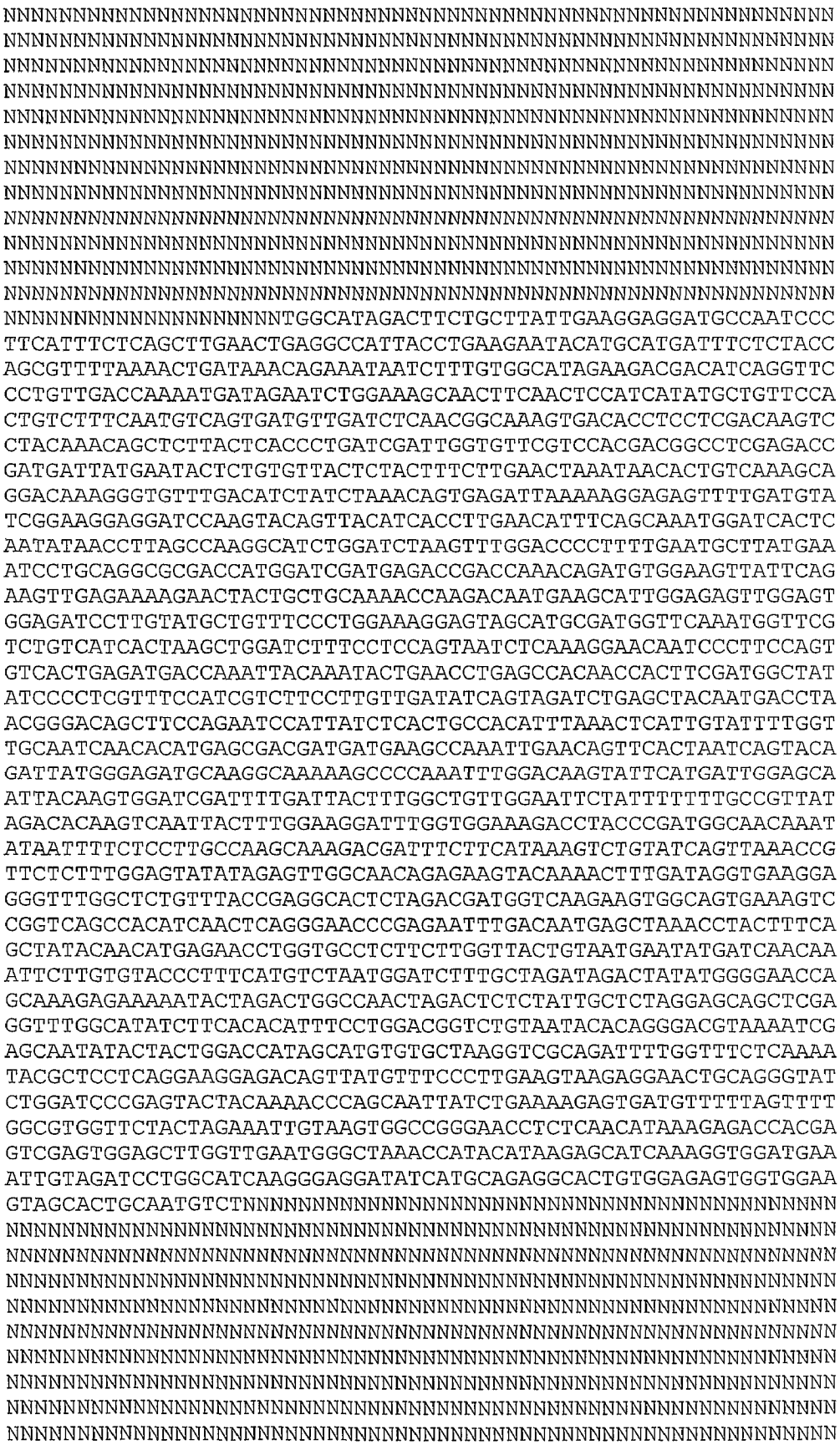
NNNN

Figure 30.

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XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
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SFGVVLLEIVSGREPLNIKRPRVEWSLVEWAKPYIRASKVDEIVDPGIKGGYHAEALWRV
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Figure 31.

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Figure 32.

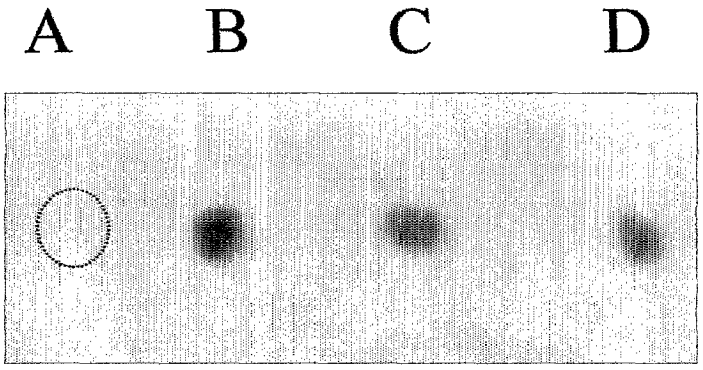

Table 1.

Name of the F2 families	Number of the F2 seeds	Number of the germinated F2 seeds	Number of the died F2 plants	Number of the Nod^- F2 plants	Number of the Nod^+ F2 plants	Nod^- : Nod^+ ratio
NAB	5571	4988	2412	50	2526	1 : 50
NBW	1285	1199	460	13	726	1 : 55

Table 2.

F2 → individuals	N B W	N B W	N B W	N B W	N B W	N B W	N B W	N B W	N B W	N B W	N B W	N B W	N B W	N B W	N B W	N B W	N B W	N B W	N B W	N B W	N B W	N B W	N B W	N B W	N B W	N B W	N B W	N B W	N B W	N B W
Name of ↓ markers	6	20	37	162	210	790	708	717	11	36	177	743	767	9	23	298	1	2	3	4	7	13	14	19	21	30	42	74	206	243
OPW8a																														
OPE8c	3	3	3	3	3	3	3	3	3	3	3	3	3	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
nn1	3	3	3	3	3	3	3	3	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
OPB13b	3	3	3	3	3	3	3	3			5	5	5	3	3	3	5	5	5	5	5					5	5	5	5	5
OPA6a	3	3	3	3	3	3	5	5	5	5	5	5	5	3	3	3	5	5	5	5	5	5	5	5	5	5	5	5	5	5

F2 → individuals	N A B	N A B	N A B	N A B	N A B	N A B	N A B	N A B	N A B	N A B	N A B	N A B	N A B	N A B	N A B	N A B	N A B	N A B	N A B	N A B	N A B	N A B	N A B	N A B	N A B	N A B	N A B	N A B	N A B	N A B
Name of ↓ markers	267	637	646	765	803	809	871	897	968	1241	908	4156	65	701	4443	334	1219	643	300	12	23	24	82	235	438	782	814	939	1166	1280
OPW8a		3		3	3			3	3	3	5	5	5	5	5	3	3	3	5	5	5	5	5	5	5	5	5	5	5	5
OPE8c	3	3	3	3	3	3	3	3	3	3	3		3	3	3	5	5	5	5	5	5	5		5	5	5	5		5	5
nn1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
OPB13b	3	3	3	3	3	3	3	3	5	5	3	3	5	5	5	5	5	3	3	5	5	5	5	5	5	5	5	5	5	5
OPA6a																														

Table 3.

A.

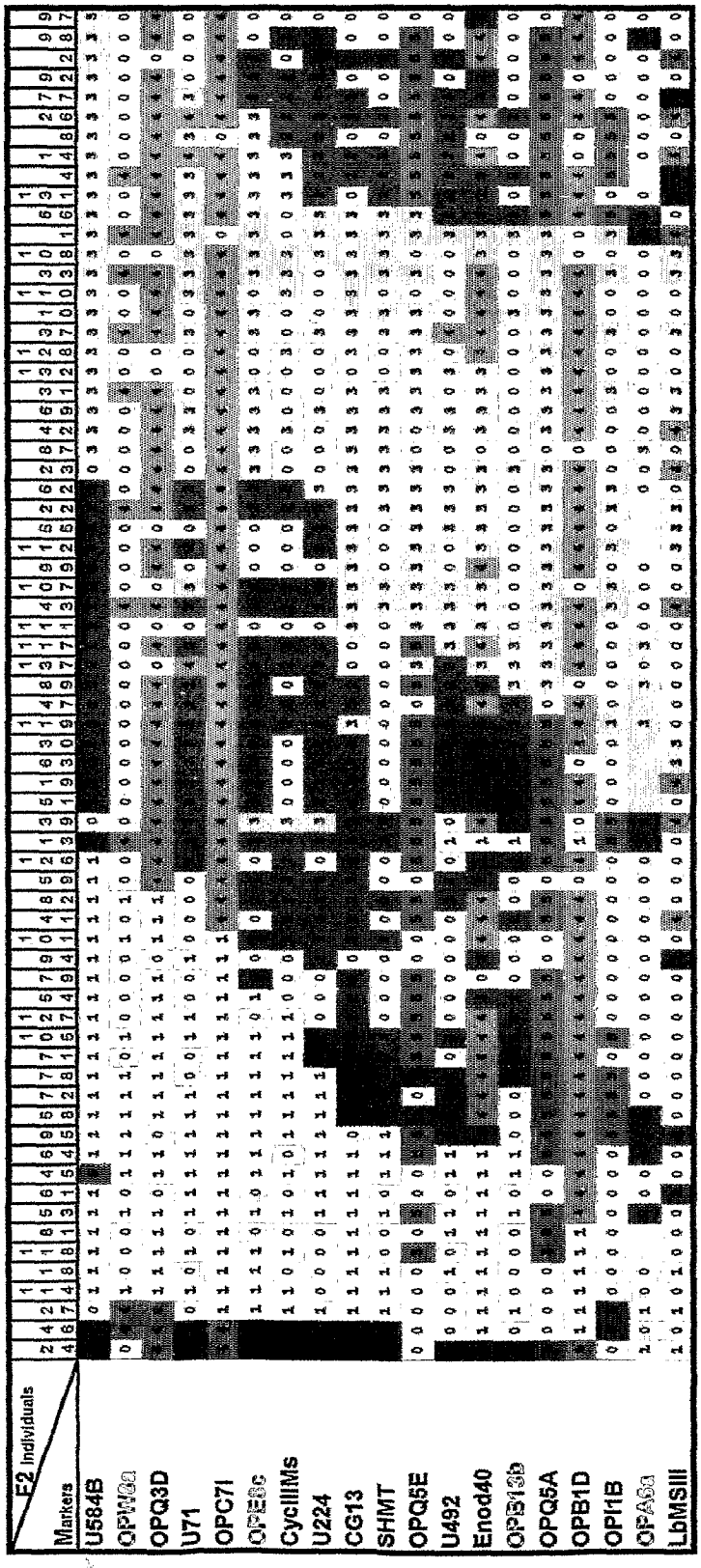
F2 Individuals
Markers
U584B
OPN02
OPQ3D
U71
OPC7I
OPE8c
CycIIIMs
U224
CG13
SHMT
OPQ5E
U492
Enod40
OPB13b
OPQ5A
OPB1D
OPI1B
OPA8a
LbMSIII

Table 3. (cont.)

B.

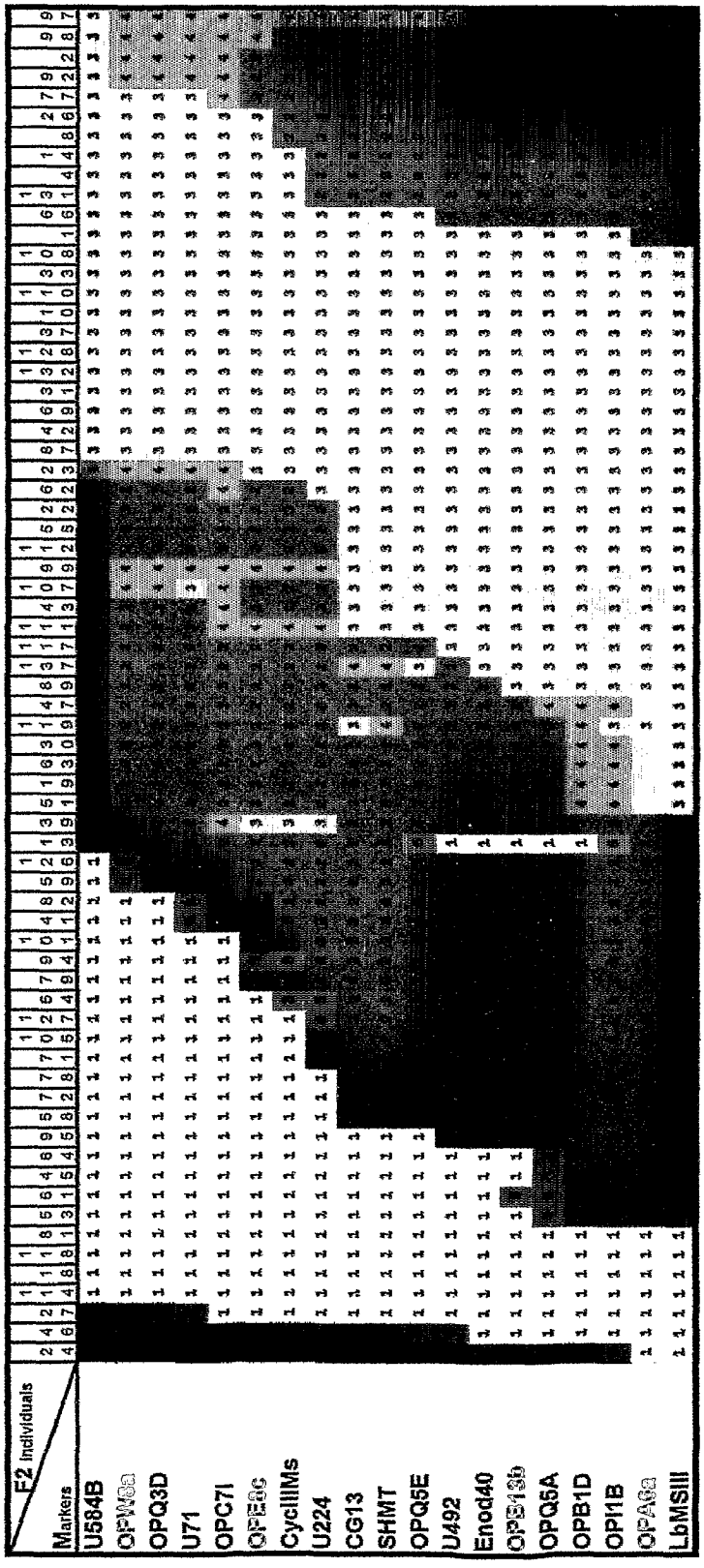

Table 4.

F2 individuals / Name of the markers	NAB 287	NAB 637	NAB 646	NAB 765	NAB 803	NAB 809	NAB 871	NAB 897	NAB 968	NAB 1241	NAB 908	NAB 4156	NAB 65	NAB 701	NAB 4443	NAB 334	NAB 1219	NAB 643	NAB 300	NAB 12	NAB 23	NAB 24	NAB 82	NAB 235	NAB 438	NAB 782	NAB 814	NAB 939	NAB 1166	NAB 1280
OPW8a		3		3	3			3	3	3	5	5	5	5	5	3	3	3	5	5	5	5	5	5	5	5	5	5	5	5
U71	3	3	3	3	3	3	3	3	3	3	3		3	3		5		5	5			5				5	5	5	5	
OPE8c	3	3	3	3	3	3	3	3	3	3	3		3	3	3	5	5	5	5	5	5	5		5	5	5	5		5	5
U224		3										5	3	3	3				5							5	5		5	
CG13	3								3	3	3	5	3	3	3						5		5			5	5	5	5	5
SHMT									3	3	3			3	3		5	5	5	5	5	5				5	5		5	
nn1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Q5E	3	3	3									3	5	3	5		5	5	5	5	5		5			5	5	5	5	5
U492	3	3	3	3	3	3	3	3	3	3	3	3	5	5	5	5	5			5	5		5	5	5	3	5	5	5	5
OPB13b	3	3	3	3	3	3	3	3	5	5	3	3	5	5	5	5	5	3	3	5	5	5	5	5	5	5	5	5	5	5

Table 5. (1/4)

28I12

NAME OF THE CLONE	RESTRICTION ENDONUCLEASE	Insert size (kb)
G18P1	Hind III	1,9
G18P4	Hind III	3,8
G18P9	Hind III	3,6
G18P17	Hind III	4,0
G18P24	Hind III	1,0
G18P31	XbaI	3,4
G18P32	XbaI	3,8
G18P37	XbaI	2,4
G18P65	EcoRI	1,0
G18P71	EcoRI	2,8
G18P75	BamHI	1,4

50E23

NAME OF THE CLONE	RESTRICTION ENDONUCLEASE	Insert size (kb)
G13P1	random	>0,5
G13P2	random	>0,5
G13P3	random	>0,7
G13P4	random	>0,8
G13P5	random	>0,7
G13P6	random	>0,7
G13P7	random	>1,0
G13P8	random	1,5
G13P9	random	1,3
G13P10	random	0,9
G13P11	random	1,5
G13P16	random	0,8
G13P32	random	1,5
G13P33	random	>0,4
G13P41	random	1,6
G13P42	random	1,3
G13P45	random	1,0
G13P47	random	1,0
G13P49	random	2,6
G13P50	random	>0,5
G13P51	random	>0,6
G13P53	random	1,3
G13P55	random	1,4
G13P58	random	1,0
G13P59	random	>1,0
G13P60	random	>0,5
G13P61	random	1,3
G13P62	random	1,1
G13P64	random	1,3

50E23

NAME OF THE CLONE	RESTRICTION ENDONUCLEASE	Insert size (kb)
G13P65	random	1,5
G13P66	random	1,6
G13P68	random	1,6
G13P69	random	>0,5
G13P70	random	1,4
G13P71	random	1,4
G13P72	random	1,4
G13P73	random	1,5
G13P74	random	>0,6
G13P75	random	1,2
G13P76	random	1,6
G13P77	random	1,6
G13P78	random	1,7
G13P79	random	1,6
G13P80	random	>0,6
G13P81	random	>0,5
G13P82	random	1,3
G13P83	random	1,2
G13P84	random	>0,1
G13P85	random	1,2
G13P86	random	>0,7
G13P87	random	1,3
G13P88	random	>0,6
G13P89	random	>0,8
G13P92	random	>0,8
G13P94	random	1,2
G13P95	random	>0,3
G13P96	random	>0,8
G13P97	random	0,8

Table 5. (cont. 2/4)

50E23

NAME OF THE CLONE	RESTRICTION ENDONUCLEASE	Insert size (kb)
G13P98	random	>0,6
G13P99	random	1,4
G13P100	random	0,2
G13P101	random	0,5
G13P104	random	1,5
G13P105	random	6,0
G13P106	random	1,4
G13P107	random	0,7
G13P108	random	6,1
G13P110	random	0,8
G13P113	random	0,1
G13P114	random	0,3
G13P115	random	0,1
G13P116	random	0,7
G13P117	random	0,7
G13P119	random	1,5
G13P120	random	0,8
G13P121	random	1,2
G13P122	random	1,6
G13P123	random	0,8
G13P126	random	1,5
G13P127	random	1,5
G13P128	random	1,0
G13P134	random	1,3
G13P138	random	1,1
G13P153	random	1,3
G13P156	random	1,1
G13P157	random	1,2
G13P158	random	1,7

50E23

NAME OF THE CLONE	RESTRICTION ENDONUCLEASE	Insert size (kb)
G13P159	random	>0,5
G13P161	random	1,3
G13P165	random	>0,6
G13P166	random	1,5
G13P167	random	1,3
G13P168	random	0,6
G13P169	random	>0,9
G14P1	Cla deléció	3,8

2D11

NAME OF THE CLONE	RESTRICTION ENDONUCLEASE	Insert size (kb)
G3P17	random	1,6
G3P46	random	0,8
G3P71	random	1,2
G3P73	random	1,2
G3P74	random	1,7
G3P91	random	1,5
G3P92	random	0,9
G3P99	random	>1,7
G3P105	random	1,7
G3P107	random	2,3
G3P123	random	1,3
G3P126	random	1,5
G3P136	random	1,0
G3P139	random	1,3
G3P147	random	1,1
G3P168	random	1,4
G3P178	random	1,5
G3P207	random	0,9
G3P222	random	1,1
G3P224	random	1,2
G3P239	random	1,1
G3P245	random	0,6
G3P268	random	0,8
G3P287	random	0,3
G3P289	random	0,9
G3P301	random	0,9
G3P411	random	1,1
G3P413	random	0,6
G3P477	random	0,9

Table 5. (cont. 3/4)

2D11

NAME OF THE CLONE	RESTRICTION ENDONUCLEASE	Insert size (kb)
G3P483	random	>1,7
G3P499	random	0,9
G3P512	random	>0,5
G3P514	random	>0,7
G3P555	random	0,7
G3P576	random	1,2
G3P578	random	0,7
G5P1	BamHI	1,5
G5P4	BamHI	1,0
G5P10	BamHI	3,1
G5P14	BamHI	3,2
G6P3	Hind III	1,7
G6P4	Hind III	1,6
G6P6	Hind III	2,7
G6P11	Hind III	0,8
G6P14	Hind III	1,7
G6P15	Hind III	0,5
G6P17	Hind III	1,8
G6P18	Hind III	1,6
G6P21	Hind III	0,3
G6P26	Hind III	1,8
G10P4	Hind III	2,7
G10P8	Hind III	2,7?
G10P25	Hind III	4,0
G11P1	Hind III	3,3
G11P9	Hind III	9,1?
G11P13	Hind III	9,0
G11P17	Hind III	6,0
G11P20	Hind III	3,3
G11P24	Hind III	8,0

2D11

NAME OF THE CLONE	RESTRICTION ENDONUCLEASE	Insert size (kb)
G11P34	Hind III	4,4
G11P36	Hind III	7,4
G11P37	Hind III	0,6
G11P38	Hind III	4,7
G11P45	Hind III	2,5
G19P21	Taq deletion	0,9
G19P27	Taq deletion	0,6
G19P42	Taq deletion	0,1
G19P46	Taq deletion	0,8
G19P48	Taq deletion	0,8
G19P73	Taq deletion	0,8
G19P74	Taq deletion	0,7
G19P81	Taq deletion	0,6
G19P94	Taq deletion	0,8

67A11

NAME OF THE CLONE	RESTRICTION ENDONUCLEASE	Insert size (kb)
G9P13	random	0,8
G9P43	random	1,1
G9P62	random	1,0
G20P1	NheI	0,9
G20P2	NheI	9,6
G20P4	NheI	6,5
G20P5	NheI	8,8
G20P7	NheI	4,4
G20P14	NheI	1,4
G20P15	NheI	5,6
G20P21	NheI	0,4
G20P31	NheI	5,0
G20P38	NheI	1,7
G20P56	NheI	12,0
G20P140	NheI	5,5
G21P25	XbaI	8,6
G21P26	XbaI	4,2
G21P34	XbaI	6,5
G21P35	XbaI	2,2
G21P43	XbaI	3,3
G21P90	XbaI	2,0
G21P136	XbaI	2,6
G21P186	XbaI	6,5
G21P319	XbaI	5
G21P448	XbaI	3,5
G22P2	PaeI-TaqI del.	3,8
G22P5	PaeI-TaqI del.	0,9
G22P7	PaeI-TaqI del.	4,1
G22P8	PaeI-TaqI del.	2,1
G22P19	PaeI-TaqI del.	4,5

Table 5. (cont. 4/4)

67A11

NAME OF THE CLONE	RESTRICTION ENDONUCLEASE	Insert size (kb)
G23P4	SacI-TaqI del.	6,1
G23P5	SacI-TaqI del.	3,3
G23P17	SacI-TaqI del.	7,2
G23P23	SacI-TaqI del.	2,6
G24P245	random	1,0
G24P413	random	0,9
G24P418	random	1,1
G24P442	random	1,1
G29P10	Hin2I	1,5
G29P23	Hin2I	0,7
G29P96	Hin2I	4,5
G29P117	Hin2I	0,8
G29P141	Hin2I	0,9
G30P193	Hin6I	6,3
G31P81	TaiI	5,5
G32P68	TaqI	0,4
G32P90	TaqI	0,3
G32P96	TaqI	1,8
G32P112	TaqI	2,7
G32P126	TaqI	5,0
G32P194	TaqI	0,5
G32P286	TaqI	3,3
G33P16	EcoRI	6,0
G33P48	EcoRI	2,3
G33P63	EcoRI	5,0
G33P64	EcoRI	3,0
G33P121	EcoRI	4,5
G33P155	EcoRI	1,6
G34P20	HindIII	1,4
G34P30	HindIII	7
G34P34	HindIII	2,3

67A11

NAME OF THE CLONE	RESTRICTION ENDONUCLEASE	Insert size (kb)
G34P42	HindIII	1,8
G34P44	HindIII	4,2
G34P46	HindIII	2,8
G34P52	HindIII	4,6
G34P64	HindIII	2,6
G34P101	HindIII	0,6
G34P173	HindIII	2,2
G34P192	HindIII	3,0
G35P65	Sau3AI	0,7
G35P85	Sau3AI	1,4
G35P136	Sau3AI	1,3
G50P4	KpnI deletion	3,5

Table 6.

Structural element	First nucleotide Position	Last nucleotide Position
EXON 1	<897	1248
EXON 2	1564	2074
EXON 3	2798	3273
EXON 4	3372	3522
EXON 5	3800	3871
EXON 6	3979	4047
EXON 7	4519	4590
EXON 8	4702	4773
EXON 9	4860	5032
EXON 10	5542	5768
EXON 11	5912	6037
EXON 12	6110	6178
EXON 13	6797	6986
EXON 14	7340	7472
EXON 15	7562	8236<

INTRON 1	1249	1563
INTRON 2	2075	2797
INTRON 3	3274	3371
INTRON 4	3523	3799
INTRON 5	3872	3978
INTRON 6	4048	4518
INTRON 7	4591	4701
INTRON 8	4774	4859
INTRON 9	5033	5541
INTRON 10	5769	5911
INTRON 11	6038	6109
INTRON 12	6179	6796
INTRON 13	6987	7339
INTRON 14	7473	7561

Table 7.

NAME OF THE CLONE	CLONING VECTOR	ENZYMES	MODIFICATION	NORK FRAGMENT	ANTIBIOTIC RESISTANCE (μg/ml)	Name of the *E. coli* strain
pBRC1667	pAT680	*Kpn* I	dephosphorilation	8.8 kb NheI	kanamycin (200)	BRC1667
pBRC1678	pPK459	*Kpn* I	dephosphorilation	8.8 kb NheI	tetracycline (10) kanamycin (50)	BRC1678
pBRC1666	pPR97	*Cla* I-*Sac* I	dephosphorilation	8.8 kb NheI	kanamycin (200)	BRC1666
pBRC1701	pPR97	*Cla* I	dephosphorilation	11.8 kb ClaI	kanamycin (200)	BRC1701

Table 8.

Agrobacterium strain	TRANSFORMED PLASMID	NAME OF THE STRAIN CARRYING THE NORK GENE
A. rhizogenes A4	pBRC1667	BRC1673
A. rhizogenes 1334	pBRC1667	BRC1675
A. rhizogenes 1724	pBRC1667	BRC1679
A. tumefaciens LBA4404	pBRC1667	BRC1677
A. tumefaciens LBA4404	pBRC1678	BRC1680
A. tumefaciens LBA4404	pBRC1666	BRC1681
A. tumefaciens LBA4404	pBRC1701	BRC1707

Table 9.

NAME OF THE STRAINS CARRYING THE NORK GENE	NUMBER OF THE PLANTS DEVELOPING ROOTS	NUMBER OF THE GUS + ROOTS	NUMBER OF THE NOD + PLANTS
BRC1673	78 (5)	1 (0)	0
BRC1675	87 (2)	3 (0)	0

Table 10.

Plants to be transformed	Number of shoots or leaves treated with Agrobacterium	Number of regenerated plants	Number of transformed plants	Number of Nod^+ plants
F1RN28	157	19	2	2
F1RN41	133	14		
F2RN28/4	120			
F2RN28/5	75			

Table 11.

Plants to be transformed	Number of shoots or leaves treated with Agrobacterium	Total number of transformants	Number of independent transformants/ Total number of transformants	Presence of the M. truncatula NORK sequence/Number of independent transformants	Presence of the NORK protein/Number of independent transformants
Nicotiana tabacum	113	15	6/15	6/6	6/6
Vicia/Lotus	232	26	8/26	8/8	nt